EXHIBIT A

Docket No. ORT 1222 USA DIV

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	David F. McComsey, et al.	
Serial No.	:	10/606,422	Art Unit: 1653
Filed	:	June 26, 2003	Examiner: David Lukton
For	:	SUBSTITUTED HETEROCYCLICS ACYL-TRIPEPTIDES USEFUL AS THROMBIN RECEPTOR MODULATORS	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF CLAUDIA K. DERIAN UNDER 37 C.F.R. 1.132

Sir:

I, CLAUDIA K. DERIAN, hereby declare as follows:

(1) THAT, my professional experience is as follows:

EDUCATION:

Massachusetts Institute of Technology, Cambridge, MA
1985 - Neural and Endocrine Regulation, Ph.D.

Wellesley College, Wellesley, MA
1978 - Biology/Chemistry, A.B.

EMPLOYMENT HISTORY:

1991-present	Johnson & Johnson	
2002-present	Johnson & Johnson Pharmaceutical Research & Development	
1991-2002	R.W. Johnson Pharmaceutical Research Institute Spring House, PA 19477	
	2000-present	Research Fellow, Drug Discovery, Vascular Research
	1991-1992	Sr. Research Scientist, Drug Discovery, Research Imaging
	1993-1999	Principal Scientist, Drug Discovery, Vascular Research
1988 - 1991	Rorer Central Research Rhone-Poulenc Rorer Central Research King of Prussia, PA 19406	
	1988 - 1990	Research Scientist, Cardiovascular Biology
	1990 - 1991	Sr. Research Scientist, Cardiovascular Biology
1985 - 1988	Merck, Sharp & Dohme Research Laboratories West Point, PA 19486	
	1985 - 1988	Postdoctoral Scientist, Blood Coagulation Laboratory, Department of Pharmacology
1980 - 1985	Massachusetts Institute of Technology Cambridge, MA 02139	
	1980 – 1985	Graduate Research Assistant Teaching Assistant, Analytical Biochemistry
	1981 1983	Teaching Assistant, Nutritional Biochemistry
1980 - 1985	Massachusetts General Hospital Boston, MA 02114	
	1980-1985	Research Assistant, Stroke Research Laboratory

1978 - 1980 Howard Hughes Medical Institute at Harvard Medical School

Boston, MA 02115
1978-1980 Senior Research Technician, Immunology

PROFESSIONAL AFFILIATIONS:

Fellow, AHA and Council on Arteriosclerosis, Thrombosis, and Vascular Biology
International Atherosclerosis Society
American Society for Biochemistry and Molecular Biology
International Society on Thrombosis and Haemostasis
The Society for Investigative Dermatology
Society for Leukocyte Biology
Sigma Xi
American Association for the Advancement of Science
New York Academy of Sciences

PATENTS AND PUBLICATIONS:

I am an author or co-author of 53 publications and 36 abstracts in the field of drug discovery.

I am an inventor or co-inventor on 8 patents or patent applications in the field of drug discovery.

HONORS AND AWARDS:

J&J COSAT Summer Intern Grant –2001
Proposal: Regulation of Protein Kinase C (PKC) in Intact Cells

Johnson & Johnson Excellence in Science Award (support for a postdoctoral fellow for 2 years) – 2001
Proposal: Transactivation Between Protein And Lipid Kinases

(2) THAT, in my present employment as a Research Fellow at Johnson & Johnson, I am responsible for conducting significant research directed towards the discovery and/or development of therapeutic agents including: originating scientific experiments; developing new methodology;

originating and developing projects; maintaining a state-of-the-art scientific knowledge; and establishing scientific credentials inside and outside the company;

(3) THAT, it is well-known in the pharmaceutical art that platelets play a major role in promoting vascular occlusion via platelet aggregation and subsequent thrombosis culminating in clinical disorders such as arterial thrombosis, venous thrombosis, acute myocardial infarction, ischemic attacks, angina, stroke and reocclusion following interventions such as thrombolytic therapy or angioplasty (Chesebro et al., *Circulation,* **86**, [suppIII] 100-110, 1992; Verstraete and Zoldhelyi, *Drugs*, **49**, 856-884, 1995; White, HD, *Am Heart J*, **138**, S570-S576, 1999; Weksler, *Cerebrovasc Dis*, **10**, 41-48, 2000, attached hereto in Exhibit B);

(4) THAT several platelet aggregation inhibitors have been tested *in vitro* and *in vivo* and have now been developed as marketed products for the treatments of thrombotic disorders. For example, clopidogrel (e.g., **Plavix®**), inhibits platelet aggregation by blocking the ADP receptor on platelets (Verstraete and Zoldhelyi, *ibid.*). Clopidogrel can inhibit platelet aggregation in animal models preventing thrombus formation. Based on these studies, it has received marketing approval to treat myocardial infarction, stroke, vascular occlusive diseases such as peripheral arterial disease and angina in patients being treated with cornonary interventions, such as angioplasties, including stents;

(5) THAT another example includes the class of fibrinogen receptor antagonists known as glycoprotein IIB/IIIa antagonists. This protein is expressed on the surface of platelets and is the common pathway by which fibrinogen binds and induces platelet aggregation. Blockage of this receptor prevents platelet aggregation. Compounds have been described in the literature that bind this receptor *in vitro*, in animal models of thrombosis, and in clinical studies. Several compounds of this class are now marketed for the treatment of thrombosis related disorders. For example, the following compounds are currently approved and marketed products: **ReoPro®** (abciximab), **Integrilin®** and **Aggrastat®** (tirofiban). In general, all three agents are approved to treat acute coronary syndromes (unstable angina), to be used during interventional

procedures such as angioplasty (Mousa, *Drug Discovery Today*, **4,** 552-561, 1999, attached hereto in Exhibit B);

(6) THAT I participated in *in vivo* studies conducted in Johnson & Johnson's laboratories of clopidogrel in a guinea pig model of thrombosis. Clopidogrel significantly prolonged the time to occlusion compared to vehicle treated animals in a model where a thrombus was generated by endothelial injury (Fig. 1). Furthermore, that a small molecule PAR-1 antagonist, which represents a new mechanism for inhibiting platelet aggregation *in vitro*, was shown to inhibit platelet aggregation *ex vivo* in guinea pigs (Andrade-Gordon et al., J Pharmacol Exp Ther, 298, 34-42; noted on pg. 36-37 and Fig 2, attached hereto in Exhibit B). Moreover, it has been demonstrated that the PAR-1 antagonist inhibited thrombosis in both a guinea pig model of carotid injury (Andrade-Gordon et al., *ibid*, *see* Fig 4 therein) and most importantly in a primate model (Derian et al, J Pharmacol Exp Ther, 304, 855-861; *see* Figs. 3, 4 and 6 therein, attached hereto in Exhibit B). These results are consistent with data known in the art demonstrating that agents which inhibit platelet aggregation and prevent *in vivo* platelet thrombus are clinically efficacious for treating platelet dependent disorders.

(7) Thus, in light of the state of the art at the time the present application was filed and the disclosure in the specification as filed, one of ordinary skill in art would understand how to use the compounds of the present application for the treatment of platelet-mediated thrombic disorders such as such as arterial thrombosis, venous thrombosis, acute myocardial infarction, ischemic attacks, angina, stroke and reocclusion following interventions such as thrombolytic therapy or angioplasty.

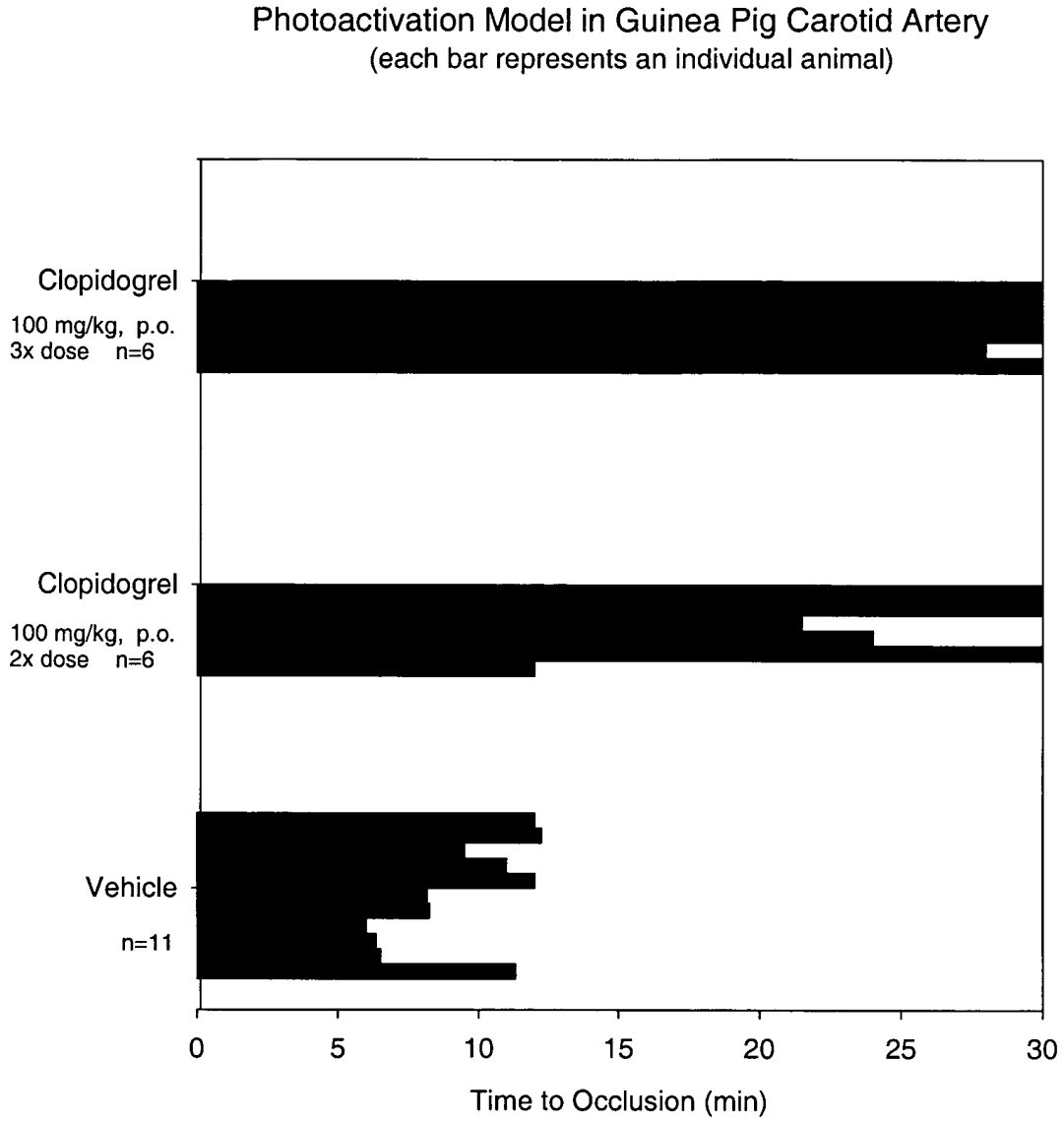

Figure 1: Inhibitory effects of Clopidogrel on thrombus formation in a guinea pig carotid thrombosis model. Each bar is an individual guinea pig response over 30 minutes. The dark bar shows the duration of patency in minutes following photoactivation with Rose Bengal dye. Guinea pigs were orally dosed for either 2 or 3 days with clopidogrel (100 mg/kg)

(8) THAT the pathophysiological processes that lead to restenosis of a blood vessel are not fully defined, however it is clear that injury to the blood vessel during clinical procedures, such as angioplasty, leads to processes including platelet aggregation, injury to the vessel causing inflammation, and induction of vascular cell proliferation (Linde and Strauss, *Expert Opin Emerging Drugs*, **6**, 281-301, 2001, attached hereto in Exhibit B). Restenosis may be prevented from occurring by inhibiting platelets from aggregating at the vessel wall surface and releasing their cellular contents. This clinical syndrome was apparent in early cases of coronary catheter injury and stent placement (Ischinger, *Am J Cardiol*, **82**; 25L-28L. 1998; Schwartz, *Am J Cardiol*, **81**, 14E-17E, 1998, attached hereto in Exhibit B).

(9) THAT the state of the art medical procedure for treating postinterventional vascular reocclusion in patients is to administer antithrombotic agents, thus preventing platelet aggregation and the release of potential agents to stimulate smooth muscle proliferation, (i.e. platelet derived growth factor) and to implant drug coated stents. Agents used clinically as antithrombotic agents include **Plavix®**, **Aggrastat®** and **ReoPro®**. Directly or indirectly preventing the stimulation of cell proliferation at the site of injury leads to the prevention of restenosis (Linde and Strauss, *ibid.*). In addition to indirectly affecting the proliferation of vascular smooth muscle cells through anti-platelet agents, more direct agents such as Sirolimus (rapamycin) or Taxol, have been shown to inhibit the proliferation of vascular smooth muscle cells (Marx, et al., *Circ Res.*, **76**, 412-417, 1995; Sollott et al., *J Clin Invest*, **95**, 1896-1876, 1995; Suzuki et al., *Circulation*, **104**:1188-1193, 2001). Both these agents are utilized clinically to prevent post-stent restenosis based on their ability to prevent vascular smooth muscle dependent proliferation.

(10) THAT a thrombin receptor antagonist for PAR-1, which is expressed on both platelets and vascular smooth muscle cells, has been shown to inhibit restenosis by inhibiting both *in vitro* vascular smooth muscle proliferation and platelet aggregation. *See* Andrade-Gordon et al. (2001) pp. 35 and 36. Inhibition of restenosis was demonstrated in an *in vivo* rat restenosis

model as shown in Fig. 6 by histology and in Table 1 by quantitative assessment. These results are consistent with previously described data demonstrating the role of inhibiting vascular smooth muscle proliferation to provide clinically efficacious results for prevention of restenosis.

(11) THAT, all statements made herein of my own knowledge are true, and all statements made on information and belief are believed to be true, and I understand that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and I understand that such willful false statements may jeopardize the validity of the application or any patents issuing thereon.

Declarant's full name is CLAUDIA K. DERIAN

DATE: 2/9/06

CLAUDIA K. DERIAN, Ph.D.

(*Circulation Research.* 1995;76:412-417.)

Articles

Rapamycin-FKBP Inhibits Cell Cycle Regulators of Proliferation in Vascular Smooth Muscle Cells

Steven O. Marx, Thottala Jayaraman, Loewe O. Go, Andrew R. Marks

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Abstract

Abstract Multiple growth factors can stimulate quiescent vascular smooth muscle cells to exit from G0 and reenter the cell cycle. The macrolide antibiotic rapamycin, bound to its cytosolic receptor FKBP, is an immunosuppressant and a potent inhibitor of cellular proliferation. In the present study, the antiproliferative effects of rapamycin on human and rat vascular smooth muscle cells were examined and compared with the effects of a related immunosuppressant, FK520. In vascular smooth muscle cells, rapamycin, at concentrations as low as 1 ng/mL, inhibited DNA synthesis and cell growth. FK520, an analogue of the immunosuppressant FK506, is structurally related to rapamycin and binds to FKBP but did not inhibit vascular smooth muscle cell growth. Molar excesses of FK520 blocked the antiproliferative effects of rapamycin, indicating that the effects of rapamycin required binding to FKBP. Rapamycin-FKBP inhibited retinoblastoma protein phosphorylation at the G1/S transition. This inhibition of retinoblastoma protein phosphorylation was

associated with a decrease in $p33^{cdk2}$ kinase activity. These observations suggest that rapamycin, but not FK520, inhibits vascular smooth muscle cell proliferation by reducing cell-cycle kinase activity.

Key Words: immunophilin • accelerated arteriosclerosis • antiproliferation • transplantation • FK506

Introduction

Abnormal vascular smooth muscle cell (VSMC) proliferation is involved in restenosis following percutaneous transluminal angioplasty (PTCA) and accelerated arteriosclerosis after cardiac transplantation.[1,2,3] Restenosis occurs after ≈30% to 40% of the procedures,[1,4] limiting the utility of PTCA. Accelerated arteriosclerosis in coronary arteries of the donor heart is a major factor limiting long-term survival of cardiac transplant recipients.[3,5,6] Common to both pathological processes is an injury to the vascular endothelial cell barrier resulting in activation of VSMC proliferation. Multiple signaling pathways can trigger a proliferative response in VSMC. The complexity of cell growth signaling has made it difficult to achieve adequate control of VSMC proliferation in patients.

Much attention has focused on understanding the mechanisms underlying the proliferative response in VSMC. It has been proposed that identifying the regulators of this proliferative response in VSMC may lead to therapeutic strategies aimed at blocking or inhibiting VSMC growth. After deendothelialization of arteries by mechanical injury during PTCA, or by an immune mechanism in transplant recipients, VSMC leave their quiescent state (G0/G1) and enter the cell cycle. Recent studies have shown that early response genes including c-*fos* and c-*myc* are induced after exit from G0.[7,8] Cell-cycle kinases including $p34^{cdc2}$ and mitogen-activated protein kinase homologues appear to be involved in signaling VSMC growth, leading to induction of early response genes.[9] On the other hand, transforming growth factor-$ß_1$ inhibits smooth muscle cells causing a G1 arrest[10,11] that is associated with a decrease in $p34^{cdc2}$ kinase activity.[12] These and other similar observations have led a number of investigators to focus on cell-cycle regulators as potential therapeutic targets for inhibiting VSMC proliferation. For example, antisense oligonucleotides to c-*myc*, c-*myb*, c-*fos*, cyclin A, $p34^{cdc2}$ kinase, and proliferating cell nuclear antigen have been used with varying degrees of success to inhibit VSMC proliferation.[11,13,14]

Recent studies in a rat heart transplantation model have suggested that the macrolide antibiotic FK506, currently used as an immunosuppressant after some types of organ transplant, may accelerate transplant coronary arteriosclerosis.[15,16,17] In animal models, rapamycin, also a macrolide antibiotic, appears to retard the development of accelerated arteriosclerosis after allograft transplantation and restenosis following mechanical injury.[15,18,19,20]

In the present study, we sought to compare the effects of rapamycin and FK520 on VSMC proliferation. We found that rapamycin, but not FK520, inhibits cell growth in both human and rat VSMC. This inhibition of cell growth by rapamycin was associated with decreases in cell-cycle kinase activity at the

G1/S and G2/M transitions. Phosphorylation of retinoblastoma protein (pRb), a marker for cell-cycle progression, was also reduced. Our data suggest that inhibition of cell-cycle kinases by rapamycin contributes to its potent antiproliferative effects in VSMC. Moreover, because the mechanism of the antiproliferative effect of rapamycin involves inhibition of cell-cycle kinases, rapamycin should block VSMC growth regardless of the stimulus that initiates the VSMC proliferative response. FK520, an analogue of FK506, nonsignificantly accelerated VSMC growth and increased the activity of the cell-cycle regulators. Thus, compared with FK520, rapamycin has antiproliferative properties that might make it a better choice for use in cardiac transplant recipients in whom VSMC proliferation is a potentially serious problem.

Materials and Methods

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Reagents

Rapamycin was a gift from Wyeth-Ayerst Research Laboratory (Dr Suren Sehgal), and FK520 (an FK506 analogue) was provided by Dr John Siekierka (Merck). [^{3}H]Thymidine was from NEN, and polyclonal anti-p34^{cdc2} kinase antibody was a gift from Dr Hiroaki Kiyokawa (Memorial Sloan-Kettering Cancer Institute). Polyclonal antibodies to cyclin D (raised against the C-terminal domain of human cyclin D) and cdk2 (raised against a synthetic peptide in the C-terminal domain of human cdk2) were from Upstate Biotechnology Inc. pRb antibody was from Pharmingen.

Cell Culture

Rat aortic smooth muscle cells (RASM, isolation No. 1120)[21] [22] [23] and human aortic smooth muscle cells were gifts from Dr Mark Taubman (Mount Sinai School of Medicine). RASM (passages 8 to 11) were cultured in Dulbecco's modified essential medium (DMEM) plus 20% fetal calf serum (FCS, GIBCO), 100 U/mL penicillin, and 100 μg/mL streptomycin as previously described.[21] Medium was changed every 48 hours. When cultured in 20% FCS, RASM double approximately every 16 to 20 hours. Human aortic smooth muscle cells from ascending aorta obtained from the donor at the time of cardiac transplantation were cultured in DMEM plus 20% FCS. After plating, rapamycin (100 ng/mL) and FK520 (100 ng/mL) were added directly to DMEM. Cell proliferation analyses were performed by counting triplicate plates at the indicated times during a 7-day period by using a Coulter counter. Cell viability was determined with trypan blue stain for each experiment. Results represent the mean values from three separate experiments; error bars represent the standard error of the mean.

DNA Synthesis

For determination of DNA synthesis, [^{3}H]thymidine incorporation was measured, and microcultures of 5000 cells were established in quadruplicates in flat-bottom 96-well microtiter plates in the presence and absence of varying concentrations of drugs. After 48 hours, each culture was pulsed with 1 μCi [^{3}H] thymidine and harvested 16 to 20 hours later by using a Cambridge Technology PHD Harvester. [^{3}H] Thymidine incorporation was measured in a liquid scintillation counter. The competition experiment with FK520 was performed with 2 ng/mL rapamycin and concentrations of FK520 between 2 and 500 ng/mL.

Flow Cytometric Analysis

Cells were treated with either 100 ng/mL rapamycin or FK520 for 24 hours, harvested, and washed in ice-cold phosphate-buffered saline (PBS), fixed in 70% ethanol, and stored overnight at 4°C before analysis. Cells were then washed once with ice-cold PBS treated with RNAse (1 hour at 37°C, 500 U/mL). Cellular DNA was stained with propidium iodide (50 μg/mL). Cell-cycle determination was performed by using a Coulter analyzer. Results represent a minimum of 3000 cells assayed for each determination.

Preparation of Cellular Lysates

RASM growing in log phase were plated at ≈30% confluence. After 24 hours in DMEM+20% FCS, plates were washed twice with PBS and transferred to DMEM+0.5% FCS for 72 hours to achieve quiescence. Plates were then stimulated with 20% FCS and treated with either no drugs (control), 100 ng/mL rapamycin, or 100 ng/mL FK520. After the indicated time period, plates were washed twice with ice-cold PBS, and cell lysates were prepared using Rb lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 120 mmol/L NaCl, 1 mmol/L EDTA, 0.1 mmol/L NaF, 0.2 mmol/L Na_3VO_4, 10 mmol/L ß-glycerophosphate, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, and 0.5% Nonidet P-40). Cells were scraped off the bottom of the plates, and lysates were rocked for 1 hour at 4°C. Lysates were stored at -70°C. Protein concentration was measured by using the Bradford reagent (Bio-Rad), with bovine serum albumin used as a standard.

Determination of Cyclin-Dependent Kinase Activities

Activities of $p34^{cdc2}$ and $p33^{cdk2}$ kinases were analyzed essentially as described previously,[24] with some modifications. Protein extracts (100 μg) were diluted to 500 μL with RIPA buffer (50 mmol/L Tris-HCl, pH 7.4, 250 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L NaF, 0.1 mmol/L Na_3VO_4, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]). RASM lysates were immunoprecipitated with either anti-$p34^{cdc2}$ kinase C-terminus–specific antiserum[25] or a human polyclonal anti-$p33^{cdk2}$ kinase antibody. Protein A–Sepharose beads (20 μL) were added and gently rocked for 1 hour at 4°C. Samples were centrifuged and washed twice with RIPA buffer, twice with RIPA without NaCl, and twice with kinase assay buffer (mmol/L: Tris-HCl 50, pH 7.4, $MgCl_2$ 10, and dithiothreitol 1). Phosphorylation was carried out in 25 μL of kinase buffer with the addition of 0.1 mg/mL of histone H1 (Boehringer Mannheim) and 50 μCi [γ-^{32}P]ATP for 15 minutes at 28°C. The reaction was terminated with the addition of 6 μL of 6x Laemmli's sample loading buffer and boiled for 5 minutes. Samples (15 μL) were analyzed on a 12% SDS-polyacrylamide gel. Gels were dried for 2 hours and analyzed by using [γ-^{32}P] ATP standards and a phosphorimager.

Measurement of Retinoblastoma Protein Levels

Protein extracts (50 μg) were size-fractionated on 7.5% gels and transferred to nitrocellulose overnight at 60 V. Filters were blocked in PBS containing 0.1% Tween-20 (PBS-T) and 5% dry milk for 1 hour at 30° C, followed by incubation overnight with anti-pRb antibody (1/1000 dilution) at 4°C. The filters were washed with PBS-T, then incubated with the secondary antibody conjugated to peroxidase (goat anti-

mouse IgG) for 1 hour at 4°C, and washed; signals were detected by using the chemiluminescence detection system (ECL) followed by exposure to Kodak XAR film. Autoradiographic signals were quantified by scanning the gels by use of a Macintosh computer with ADOBE PHOTOSHOP and IMAGE 1.44 software. The ratio of hyperphosphorylated to hypophosphorylated pRb was calculated for each time point and plotted. Results are shown for a representative experiment. This experiment was repeated three times, and similar results were obtained each time.

Measurement of Cyclin-Dependent Kinase and Cyclin Protein Levels

Protein extracts (50 μg) were electrophoresed on separate 12% SDS-polyacrylamide gels and transferred to nitrocellulose overnight at 45 V. Filters were blocked in PBS containing 0.1% Tween 20 and 5% dry milk for 1 hour at room temperature, followed by incubation overnight with anti-p34^{cdc2} antibody (1/1000 dilution), anti-p33^{cdk2} antibody (2.5 μg/mL), or anti–cyclin D antibody (2.5 mg/mL). The filters were then washed, incubated with goat anti-rabbit IgG conjugated to peroxidase for 1 hour at 4°C, and washed again; signals were detected by using the chemiluminescence detection system (Bio-Rad) and exposed to Kodak XAR films. Results are shown for representative experiments. These experiments were repeated three times, and similar results were obtained each time.

Results

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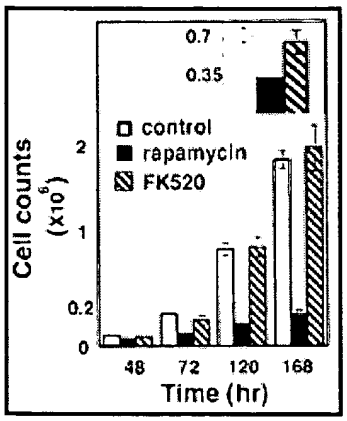
Rapamycin as low as 1 ng/mL, but not FK520 at any dose tested, inhibited RASM proliferation ($P<.05$, Fig 1). Rapamycin also decreased [^{3}H] thymidine incorporation in a dose-dependent manner ($P<.01$, Fig 2A). The inhibition of proliferation produced by rapamycin persisted at least through 7 days (168 hours) of cell culture. Similarly, rapamycin inhibited human aortic smooth muscle cell proliferation by 50% after 72 hours ($P<.05$; Fig 1, inset). In contrast, FK520 increased cell growth compared with control, but the differences were not significant. Cell viability, as assessed by trypan blue staining, was >99% in control, rapamycin-treated, and FK520-treated cells. The effect of rapamycin in terms of inhibiting DNA synthesis was competed by a molar excess of FK520 (Fig 2B). This result indicates that the reduction in [^{3}H]thymidine incorporation was probably mediated by rapamycin binding to the immunophilin FKBP, since both rapamycin and FK520 share this same cytosolic receptor. FK520 at low concentrations (eg, 2.5 ng/mL) caused a small but significant ($P<.05$) decrease in DNA synthesis (Fig 2A), and there was a small additive effect of FK520 (only at low concentrations, eg, 8 and 16 ng/mL) combined with rapamycin in terms of decreasing DNA synthesis (Fig 2B).

Figure 1. Bar graph showing the time course for rapamycin-induced inhibition of cultured rat aortic smooth muscle cell (RASM) proliferation. The inset shows similar data for human aortic smooth muscle cells at 72 hours. Cells were treated with either no drug, 100 ng/mL rapamycin, or 100 ng/mL FK520. The results are expressed in mean cell numbers of triplicate plates; error bars represent standard deviation of the mean. The results are



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representative of three similar experiments. $P<.05$ for the comparison between control and rapamycin for both RASM and human aortic smooth muscle cells at each time point after 48 hours.

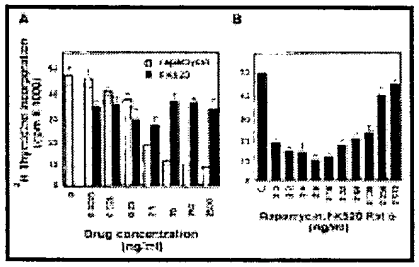

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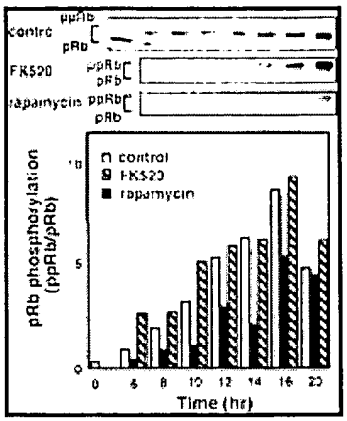
Figure 2. A, Bar graph showing the effect of immunosuppressive drugs rapamycin and FK520 on the incorporation of [^{3}H]thymidine in cultured rat aortic smooth muscle cells. B, Bar graph showing that FK520 competes with rapamycin for binding to FKBP12 and inhibits the effects of rapamycin on [^{3}H]thymidine uptake in cultured rat aortic smooth muscle cells. The results are a mean of quadruplicate wells, and the error bars represent standard deviation of the mean. $P<.05$ for the comparison between rapamycin-treated and control cells at each concentration above 0.0025 ng/mL. The results are representative of three similar experiments.

Rapamycin, but not FK520, delayed progression from G1 to S as assessed by cell-cycle analysis using propidium iodide staining. After stimulation with 20% FCS, ≈30% of cells progressed from G1/S and G2/M. The effect of rapamycin was to reduce progression from G1/S and G2/M to ≈10% of cells. pRb phosphorylation is believed to be a marker for progression from G1 to S. Hypophosphorylated pRb suppresses the progression from G1 to S,[26] and hyperphosphorylation generally occurs 1 to 2 hours before the G1/S transition.[27] [28] Cells in early G1 contain exclusively hypophosphorylated pRb. At an undefined point in late G1, pRb is hyperphosphorylated and remains hyperphosphorylated until M. In quiescent RASM (maintained in 0.5% FCS for 72 hours), pRb phosphorylation occurred 6 to 8 hours after stimulation with 20% FCS. Culturing cells with rapamycin (100 ng/mL) delayed the onset of pRb hyperphosphorylation in RASM by 6 hours to ≈12 hours after G0 and reduced the levels of phosphorylation at each time point sampled (Fig 3). In contrast, FK520 nonsignificantly accelerated the time course of pRb phosphorylation (Fig 3).

Figure 3. Bar graph showing the effects of rapamycin and FK520 on phosphorylation of retinoblastoma protein in cultured rat aortic



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smooth muscle cells. Cells were treated with either no drug, 100 ng/mL rapamycin, or 100 ng/mL FK520. The indicated times are in hours after G0. The positions indicating hyperphosphorylation and underphosphorylation (ppRb and pRb, respectively) are indicated at the left of each gel panel (inset above graph). The results shown are from a representative experiment. Similar results were obtained in three experiments.

Progression through the cell cycle is dependent on the activity of specific cell-cycle kinases, several of which, including cdk2 and cdk4, are thought to phosphorylate pRb. We examined the effects of rapamycin and FK520 on the activity of several cell-cycle kinases in VSMC. In RASM compared with control cells, $p34^{cdc2}$ kinase activity was decreased ≈16 to 20 hours after G0 by rapamycin but not by FK520 (Fig 4A). Protein levels of $p34^{cdc2}$ kinase were unchanged throughout the cell cycle (Fig 4A, insets). The decrease in $p34^{cdc2}$ kinase activity at ≈16 to 20 hours corresponds to the G2/M transition in RASM. At earlier time points (during the G1/S transition), $p34^{cdc2}$ kinase activity was low despite steady levels of $p34^{cdc2}$ protein, suggesting that it may have little effect at this point in the cell cycle in RASM (Fig 4A).

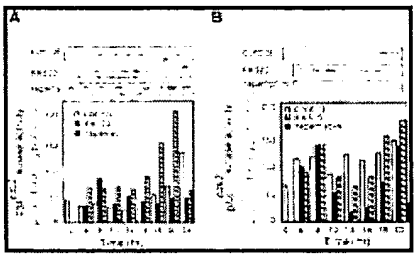

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Figure 4. A, Bar graph showing that rapamycin, but not FK520, decreases $p34^{cdc2}$ kinase activity at the G2/M transition in cultured rat aortic smooth muscle cells. The insets are immunoblots showing $p34^{cdc2}$ protein levels. Cells were treated with either no drug, 100 ng/mL rapamycin, or 100 ng/mL FK520. B, Bar graph showing that rapamycin, but not FK520, decreases $p33^{cdk2}$ kinase activity at the G1/S transition. The insets are immunoblots showing $p33^{cdk2}$ protein levels. With the exception of the earliest two time points in the control samples, the level of $p33^{cdk2}$ protein remained constant throughout the cell cycle. The results shown are from representative experiments. Similar results were obtained in three experiments.

Compared with control cells, rapamycin (100 ng/mL) decreased $p33^{cdk2}$ kinase activity beginning at 10 hours through 16 hours after G0 (Fig 4B). The period from 10 to 16 hours after G0 corresponds to the time during which pRb phosphorylation is decreased by rapamycin-FKBP (Fig 3). Protein levels for $p33^{cdk2}$ kinase were unchanged throughout the cell cycle compared with control cells (Fig 4B, insets),

indicating that the decrease in $p33^{cdk2}$ kinase activity was not due to a decrease in $p33^{cdk2}$ synthesis. These data suggest that the inhibition of pRb phosphorylation could at least in part be due to a decrease in $p33^{cdk2}$ kinase activity.

A regulatory role for cyclin D1 has been proposed with regard to pRb phosphorylation.[29] [30] Interactions between cyclin D1 and a variety of cyclin-dependent kinases have been reported, and the expression of D-type cyclins is regulated by growth factors.[31] We sought to determine, on the basis of these observations, whether the antiproliferative effects of rapamycin in RASM were associated with regulation of cyclin D1. Cyclin D1 levels were elevated in control RASM at ≈10 hours after G0, corresponding to the onset of pRb phosphorylation. Rapamycin delayed the onset of this rise in cyclin D1 levels by 4 to 6 hours (data not shown). The reduction in cyclin D1 levels by rapamycin occurred at the point when pRb phosphorylation was reduced.

Discussion

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Our data show that the antiproliferative effects of rapamycin in VSMC are associated with an inhibition of cell-cycle kinases, cyclins, and pRb phosphorylation. These data imply that phosphorylation of pRb plays an important role in signaling during smooth muscle proliferation. In contrast, FK520, another potentially useful drug for immunosuppression following cardiac transplantation, induces a nonsignificant increase in VSMC proliferation associated with an acceleration of the time course and extent of pRb phosphorylation (Figs 1⊞ and 3⊞). The antiproliferative effects of rapamycin appear to be mediated by binding to the cytosolic receptor FKBP because they are competed by FK520, a drug that shares the same receptor. However, these studies do not exclude the possibility that these drugs also interact with other binding sites in RASM.

We observed small but significant effects only at low concentrations of FK520 (2 to 20 ng/mL) in terms of inhibiting DNA synthesis. However, the physiological importance of these effects was questionable because we never observed any inhibition of RASM proliferation when using either FK520 or FK506 at any concentration. Indeed, to the contrary, we have consistently observed a small nonsignificant increase in proliferation when using either FK520 or FK506. Moreover, FK520 increases pRb phosphorylation and cell-cycle kinase activity (Figs 3⊞ and 4⊞), suggesting that it accelerates cell-cycle progression.

Cyclin-dependent kinases, including cdk2, have been implicated as regulators of pRb function.[32] [33] [34] Although the data suggest that a cyclin-dependent kinase phosphorylates pRb in vitro, there remains some controversy as to which kinase and how many actually carry out this function in vivo. We found that rapamycin but not FK520 decreased the activity of $p33^{cdk2}$ kinase[35] in VSMC. This finding does not indicate that pRb phosphorylation is dependent on $p33^{cdk2}$ kinase but suggests that this kinase could be involved in regulating pRb function in VSMC. We did not examine the activities of other kinases that similarly could be playing a role in phosphorylating pRb. Indeed, determining the specific kinase(s) that phosphorylates pRb would be interesting but is not required to support the main point of the present study, which is that the antiproliferative effects of rapamycin in VSMC are associated with inhibition of

regulators of cell-cycle progression. Similarly, the finding that rapamycin decreased the activity of cell-cycle kinases and the levels of a cyclin (D1) does not rule out the possibility that rapamycin could have effects on other regulators of cell growth as well.

$p34^{cdc2}$ kinase is thought to play an important regulatory role in the G2/M transition.[36] [37] The time course for inhibition of $p34^{cdc2}$ kinase activity by rapamycin suggests that this kinase may play an important role in the G2/M transition in VSMC. In another myogenic cell line, BC3H1 cells, we had previously shown that rapamycin inhibited proliferation and induced differentiation and that these effects were also associated with a reduction in $p34^{cdc2}$ kinase activity.[24] However, in BC3H1 cells, the decrease in $p34^{cdc2}$ kinase activity occurs at the G1/S transition. The $p34^{cdc2}$ kinase may have multiple roles in the cell cycle, depending on which cell type is examined.

The growth-inhibitory effects of rapamycin (Fig 1) in VSMC are long lasting. In contrast, inhibition of pRb phosphorylation (Fig 3) and cell-cycle kinase activity (Fig 4) appears to be more of a transient delay rather than a complete block. Moreover, examination of the cell growth data in Fig 1 shows that although growth is significantly suppressed by rapamycin, there is some slow growth in the rapamycin-treated cells. Indeed, taken together, these data suggest that rapamycin significantly lengthens the cell cycle by introducing delays at the G1/S and G2/M transition points. These delays appear to result in a marked prolongation of the doubling time for the VSMC exposed to rapamycin (Fig 1). Some of the cell-cycle kinase activity and phosphorylation of pRb observed later in the cell cycle (eg, at 16 to 20 hours) in the rapamycin-treated cells may reflect the fact that the cell cultures were not synchronized. Thus, a subset of cells was past the G1/S transition at the start of the experiment, despite culturing in low-serum medium for 72 hours to induce quiescence.

It is believed that immunologic events linked to HLA incompatibility between the donor and host may result in vascular injury, leading to VSMC proliferation. Moreover, accelerated arteriosclerosis is not limited to cardiac transplant patients, as other organ allografts are subject to similar processes.[38] Cyclosporin A, one of the most widely used immunosuppressants, appears to have a neutral effect on accelerated arteriosclerosis.[6] [15] [38] [39] The mechanisms underlying post–cardiac transplant–accelerated arteriosclerosis remain poorly understood.[5] [15] [38] [40] Nevertheless, VSMC proliferation is the fundamental pathology. Accelerated arteriosclerosis after cardiac transplantation occurs with similar frequency despite the use of newer immunosuppressant agents, including cyclosporin A and FK506. FK506 is currently being used as a therapeutic agent for the prevention of post–cardiac transplant rejection in humans. Our findings predict that FK506 would either be neutral in terms of VSMC proliferation or could have an adverse effect by accelerating the time course and the extent of posttransplant arteriosclerosis. Conversely, since rapamycin both immunosuppresses and blocks VSMC proliferation, it could be the preferred therapeutic agent to reduce accelerated arteriosclerosis following cardiac transplantation and might even prolong survival in cardiac transplant recipients.

Many studies have attempted to identify the factor or factors contributing to VSMC proliferation following vascular injury, particularly after PTCA.[2] [9] [12] [21] [22] [41] [42] [43] [44] [45] [46] The fact that rapamycin inhibits cell-cycle dependent kinases and phosphorylation of pRb suggests that its effects on VSMC

proliferation would not depend on which of the many agents capable of triggering VSMC proliferation after injury are causative. As such, rapamycin might also be a useful agent for reducing or blocking the component of post-PTCA restenosis that is due to VSMC proliferation.

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Taxol Inhibits Neointimal Smooth Muscle Cell Accumulation after Angioplasty in the Rat

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Abstract

Despite significant improvements in the primary success rate of the medical and surgical treatments for atherosclerotic disease, including angioplasty, bypass grafting, and endarterectomy, secondary failure due to late restenosis continues to occur in 30–50% of individuals. Restenosis and the later stages in atherosclerotic lesions are due to a complex series of fibroproliferative responses to vascular injury involving potent growth-regulatory molecules (such as platelet-derived growth factor and basic fibroblast growth factor) and resulting in vascular smooth muscle cell (VSMC) proliferation, migration, and neointimal accumulation. We show here, based on experiments with both taxol and deuterium oxide, that microtubules are necessary for VSMCs to undergo the multiple transformations contributing to the development of the neointimal fibroproliferative lesion. Taxol was found to interfere both with platelet-derived growth factor–stimulated VSMC migration and with VSMC migration and with VSMC proliferation, at nanomolar levels in vitro. In vivo, taxol prevented medial VSMC proliferation and the neointimal VSMC accumulation in the rat carotid artery after balloon dilatation and endothelial denudation injury. This effect occurred at plasma levels approximately two orders of magnitude lower than that used clinically to treat human malignancy (peak levels achieved in this model were ~ 50–60 nM). Taxol may therefore be of therapeutic value in preventing human restenosis with minimal toxicity. (*J. Clin. Invest.* 1995. 95:1869–1876.) Key words: restenosis • atherosclerosis • microtubules • tubulin • deuterium oxide

Introduction

Microtubules, ubiquitous cellular constituents present in all eukaryotic cells, are required for normal cellular activities. They are an essential component of the mitotic spindle needed for cell division and are required for maintaining cell shape and a variety of other cellular activities, such as motility, anchorage, transport between cellular organelles, extracellular secretory processes (1), modulation of the interactions of growth factors with cell surface receptors, and intracellular signal transduction (2–5). Furthermore, microtubules probably play a critical regulatory role in cell replication, as both the c-*mos* oncogene and CDC-2-kinase, which regulate entry into mitosis, bind to and phosphorylate tubulin (6, 7) and the product of both the tumor suppressor gene p53 and the T-antigen of simian virus 40 bind tubulin in a ternary complex (8). Microtubules are in dynamic equilibrium with their soluble protein subunits, the α- and β-tubulin heterodimers (1). Assembly under physiologic conditions requires GTP and certain microtubule-associated and -organizing proteins as cofactors; on the other hand, high calcium and cold temperature cause depolymerization.

Interference with this normal equilibrium between the microtubule and its subunits would therefore be expected to disrupt cell division and motility, as well as other activities dependent on microtubules. This strategy has been used with significant success in the treatment of certain malignancies. Indeed, antimicrotubule agents are among the most important anticancer drugs currently used. The vinca alkaloids, which promote microtubule disassembly, play principal roles in the chemotherapy of most curable neoplasms, including acute lymphocytic leukemia, Hodgkin's and non-Hodgkin's lymphomas, and germ cell tumors, as well as in the palliative treatment of many other cancers.

The newest and most promising antimicrotubule agent under research is taxol. Taxol, the prototype of the taxane class of compounds, is a highly derivatized diterpenoid (9) isolated from the bark from the Pacific yew, *Taxus brevifolia.* Taxol induces tubulin polymerization, resulting in the formation of abnormally stable and nonfunctional microtubules (10, 11). This is a novel mechanism compared with classic antimicrotubule agents such as colchicine and the vinca alkaloids, which induce microtubule disassembly. Taxol has one of the broadest spectra of antineoplastic activity, renewing serious interest in chemotherapeutic strategies directed against microtubules (for review, see reference 12). In recent phase II studies, taxol has shown significant activity in advanced and refractory ovarian cancer (13, 14) and malignant melanoma (15), as well as in cancers of the breast (16), head and neck, and lung.

Despite significant improvements in the primary success rate of the medical and surgical treatments for atherosclerotic disease, including angioplasty, bypass grafting, and endarterectomy, secondary failure due to late restenosis continues to occur in 30–50% of individuals. During angioplasty, intraarterial balloon catheter inflation results in deendothelialization, disruption of the internal elastic lamina, and probably injury to some medial smooth muscle cells. Although restenosis after such vascular injury likely results from the interdependent actions of the ensuing thrombosis, inflammation, elaboration of potent growth-regulatory molecules (such as PDGF basic fibroblast

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growth factor), and smooth muscle cell accumulation (17), the final common pathway evolves as a result of vascular smooth muscle cell (VSMC)[1] dedifferentiation from a contractile to a secretory phenotype (18). This involves, principally, VSMC secretion of matrix metalloproteinases, which degrade the surrounding basement membrane (19), proliferation, chemotactic migration into the intima (20–27), and secretion of a large extracellular matrix, forming the neointimal fibroproliferative lesion.

Since the pattern of the VSMC phenotypic dedifferentiation after arterial injury mimics that of neoplastic cells (i.e., abnormal proliferation, growth-regulatory molecule and protease secretion, migration, and basement membrane invasion), we tested the hypothesis that interference with VSMC microtubule function by taxol would inhibit restenosis by preventing neointimal VSMC accumulation. Taxol was found to stabilize microtubule polymerization and to interfere with PDGF-stimulated intracellular signaling, proliferation, and migration, at nanomolar levels in vitro. In vivo, taxol prevented neointimal smooth muscle cell accumulation in the rat carotid artery after balloon dilatation and endothelial denudation injury at plasma levels one to three orders of magnitude lower than peak plasma levels achieved with standard doses and schedules used clinically to treat human malignancy and at pharmacologic exposures substantially lower than those associated with cytotoxic effects in the clinic. Thus, microtubule stabilization offers a potentially viable approach to preventing neointimal VSMC accumulation after injury by interfering with a diverse array of sensitive intracellular processes that are likely to have an important role in the development of restenosis. Although the biology of human restenosis may differ somewhat from that of animal models, particularly in the kinetics of cell proliferation (28, 29), the ability of taxol to alter a variety of cellular activities (including intracellular signaling, VSMC proliferation and migration, and potentially matrix production) increases the likelihood of success in preventing human restenosis with minimal toxicity over other strategies based solely on the inhibition of cellular proliferation.

Methods

Cell culture and immunocytochemistry. Rat VSMCs were isolated and cultured according to techniques previously described (30). VSMCs were isolated by collagenase/elastase enzymatic digestion of the medial layers of the rat aorta obtained from 6-mo-old Wistar rats. The cells were maintained in culture with high glucose DMEM supplemented with 10% FBS and nonessential amino acids (30). Cell cultures were maintained at 37°C in 5% CO_2. Passages 8–16 were used throughout the study. Taxol (added from a 1-mM stock solution in DMSO, Sigma Chemical Co., St. Louis, MO) was soluble in the culture media of treated cells at least to the level of 100 nM (the maximum dose tested). In other experiments requiring deuterium oxide–treated cells, ^2H_2O (99.9 atom % deuterium, low tritium; Aldrich Chemical Co., Milwaukee, WI) was substituted (vol/vol) for H_2O in the preparation of the DMEM from concentrated stock. The degree of tubulin polymerization in these cells, after pretreatment for 18 h with taxol, ^2H_2O, or control media in culture, was assessed via indirect immunostaining; cells were fixed after pretreatment in 3.7% formaldehyde and permeabilized with 1% Triton X-100. Polymerized tubulin was labeled with mouse anti-β-tubulin antibody (mAb SMI 62 against polymerized β-tubulin; Paragon Biotec, Inc., Baltimore, MD). Secondary labeling was achieved with silver-enhanced, 1-nm gold-conjugated rabbit anti-mouse antibody (Goldmark Biologicals, Phillipsburg, NJ).

In vitro chemoinvasion. Chemoinvasion assays were performed using a modified Boyden chamber (31), consisting of an upper chamber separated from a lower chamber by a porous polyvinylpyrrolidone-free filter. PVPF filters (8-μm pore diameter; Nucleopore Filters, Pleasanton, CA) were coated and air dried consecutively with solutions containing 5 μg of type I collagen, 5 μg of fibronectin, and 10 μg of reconstituted basement membrane (produced from the Englebreth-Holm-Swarm tumor [32]), producing a continuous 10-μm-thick coating of matrix material. Boyden chambers were assembled with PDGF-BB (10 ng/ml in DMEM in the lower [chemoattractant] chamber); then cells (200,000) suspended in DMEM containing 0.1% BSA were added to the upper chamber. Some of the cells used in these assays were pretreated for 18 h with taxol at concentrations of 30 pM to 100 nM in culture; these concentrations affected neither cell viability (as assessed by trypan blue exclusion) nor cell attachment onto type I collagen–coated surfaces. In the taxol-treated groups, taxol was added to the upper and lower chambers at the same concentration as that used for pretreatment. Some of the cells used in these assays were pretreated for 18 h with ^2H_2O (25, 50, or 75% [vol/vol] substitution for H_2O) in culture. In the ^2H_2O-treated groups, ^2H_2O-substituted DMEM (vol/vol) was added to the upper and lower chambers at the same concentration as that used for pretreatment. In each of these experiments, the chambers were then incubated for 4 h at 37°C in a humidified 5% CO_2 atmosphere. Insignificant cellular proliferation occurs (i.e., VSMC number remains essentially constant) during the 4-h time course of these assays. At the conclusion of the experiment, the filters were removed and the cells were fixed and stained with hematoxylin and eosin. After the cells on the upper surface of the filter (noninvaders) were mechanically removed, the cells on the underside (invaders) were counted under ×400 (four random fields were counted per filter, all experiments were run in triplicate, and each triplicate assay was repeated three times on separate occasions using different VSMC preparations). Chemotaxis was assayed in analogous fashion in the Boyden chambers previously described, except that the reconstituted basement membrane was omitted. We have previously shown that these Boyden assays specifically measure VSMC chemotaxis to a PDGF *gradient* (rather than chemokinesis), since no significant migration occurs when equal concentrations of PDGF are placed on both sides of the filter barrier or when PDGF is replaced by a nonspecific agent such as BSA (19).

Metalloproteinase activity. Gelatinase zymography was performed on the supernatants removed after the 4-h conclusion of the Boyden assays. Gelatin-degrading metalloproteinases (including the 72-kD type IV collagenase) secreted into the media by VSMCs were detected with SDS-PAGE using gels containing 0.1% gelatin. Gelatinase activity was then reconstituted in 2.5% Triton X-100 for 30 min at 23°C, followed by 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% BRIJ 30 (Sigma Chemical Co., St. Louis, MO), 50 mM Tris-HCl, pH 7.6, for 18 h at 37°C. Gelatinolytic activity was observed in gels stained with 0.5% Coomassie brilliant blue G-250 (destaining was with 10% acetic acid, 40% methanol) as a colorless band against a background of blue-stained, nondegraded gelatin.

Measurement of DNA synthesis. [^{3}H]Thymidine incorporation was measured to determine the effect of taxol on VSMC DNA synthesis. VSMCs, pretreated with the various concentrations of taxol for 18 h (and throughout the assay), were plated at 4.5×10^4 on 24-well plates. After a 5-h incubation in 10% FCS + DMEM, 0.5 mCi of [^{3}H]-thymidine was added and the incubation was continued for an additional 16 h. Cells were washed twice with PBS, extracted with 10% TCA for 2 h on ice, and centrifuged at 2,000 *g* for 10 min. Supernatants were decanted, and pellets were solubilized in 0.5 ml of 1 N NaOH. After neutralizing with 0.5 ml of 1 N HCl, [^{3}H]thymidine uptake was measured by liquid scintillation counter (Beckman Instr., Fullerton, CA). Each condition of these experiments was performed in triplicate. Incor-

1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; IC_{50}, concentration producing 50% inhibition; PDGF, platelet-derived growth factor; VSMC, vascular smooth muscle cell.

poration of the thymidine analog bromodeoxyuridine (BrdU) was measured to determine the effect of ^2H_2O on VSMC DNA synthesis. VSMCs were plated at 4.5×10^4 on 24-well plates. After a 20-h incubation in 10% FCS + DMEM at various ^2H_2O concentrations, 10 μM BrdU was added, and the incubation was continued for an additional 4 h. Cells were washed twice with PBS and fixed with 100% methanol (−20°C) for 10 min. The cells were incubated for 2 h with 1 N HCl to denature the DNA and subsequently washed four times in PBS. Mouse anti-BrdU mAb (Boehringer Mannheim, Indianapolis, IN) in 2% BSA–PBS was incubated with cells for 1 h. After washing in PBS, goat anti-mouse antibody conjugated with alkaline phosphatase was added. Cell nuclei containing BrdU substituted for thymidine stained red with alkaline phosphatase substrate, whereas all other nuclei stained blue. The fraction of BrdU-positive nuclei was compared in control (defined as 100%) and ^2H_2O-pretreated groups.

Carotid artery injury model. 6-mo-old Wistar rats were anesthetized with 20 mg/kg body weight pentobarbital, 2 mg/kg body weight ketamine, and 4 mg/kg body weight xylazine intraperitoneally under a protocol approved by the National Institute on Aging Animal Care Committee. The left external carotid artery was cannulated with a 2-French Fogarty embolectomy catheter, which was then inflated with saline and passed three times up and down the common carotid artery to produce a distending, deendothelializing injury. The animals were treated with 2 mg/kg body weight taxol in vehicle; control animals were treated with vehicle alone (13.4 ml/kg body weight per d of 1:2:2:165 DMSO/Cremephor EL (Sigma Chemical Co.)/ethanol/PBS) 2 h after the injury. Taxol or vehicle alone was administered daily for the next 4 d. In other experiments (not involving taxol), rats were equilibrated with 25% ^2H_2O drinking water for 6 wk before carotid injury (a duration estimated to result in > 23% ^2H_2O replacement of body water) and until the carotid arteries were removed for study. After 11 d, the animals were anesthetized as previously described, and the carotid artery was isolated, fixed in 10% buffered formalin, and embedded in paraffin. Cross sections of the carotids were mounted on microscope slides and stained with hematoxylin and eosin. Morphometric analysis was performed from three to four individual sections from the middle of each injured arterial segment. The image of each carotid artery section was projected onto a digitizing board (SigmaScan, Jandel Scientific, Corte Madera, CA), and the cross-sectional areas of the intima and the media were measured. Experiments were coded so that surgery and data analysis were performed without knowledge of treatment group.

Measurement of in situ VSMC proliferation. In some animals undergoing the carotid artery injury protocol, the effect of taxol versus vehicle on in situ VSMC proliferation was measured via BrdU incorporation at day 2 after injury (approximately the point of maximal VSMC proliferation rate after carotid artery balloon injury) (33). Briefly, taxol- and vehicle-treated rats were injected subcutaneously with three doses of BrdU (30 mg/kg) at 30, 38, and at 46 h after injury. The carotid artery sections were harvested at 48 h after injury, and histologic sections were incubated with mouse anti-BrdU mAbs (Boehringer Mannheim) to label BrdU incorporation. Proliferating VSMCs were identified by positive staining with the anti-BrdU antibody. The fraction of BrdU-positive medial VSMC nuclei per cross section (no neointima present at day 2) was compared between taxol and vehicle treatment groups.

Measurement of plasma taxol. Plasma was obtained from pairs of rats at 0.5, 1, 2, 3, 4, 6, and 24 h after treatment with taxol (2 mg/kg i.p.) and from two vehicle-treated animals serving as controls and was stored at −20°C. Plasma taxol concentrations were measured at The Johns Hopkins Oncology Center (Baltimore, MD) by HPLC using a modification of the method described by Longnecker et al. (34). Briefly, 15 μl of 0.1 mmol/liter internal standard (*n*-cyclohexylbenzamide) was added to a 1-ml plasma sample. Taxol and internal standard were extracted with 5 ml of ethyl acetate, and the organic layer was dried under a nitrogen stream. The residue was reconstituted with 200 μl of acetonitrile. 25 μl of the solution was injected onto the column using an autosampler. A C_{18} column, 150 × 4.6 mm, 5 μm (Jones Chromatography, Lakewood, CO), with a C_{18} Guard-Pak precolumn insert (Waters Chromatography, Marlborough, MA) was used. The separation was achieved at a flow rate of 1.5 ml/min beginning with 65% Milli-Q water (Waters Chromatography)/35% acetonitrile for 6 min, followed by a linear gradient to 35% Milli-Q water/65% acetonitrile over 17 min. Ultraviolet detection was performed at 227 nm. The retention times for the internal standard and taxol were 9 and 17.4 min, respectively. The limit of taxol quantification was 0.02 μmol/liter, the within-run analytic coefficient of variation was 3%, and the total (between-day) analytic coefficient of variation was 8.5%. Chromatographic data were acquired by and subsequently analyzed using an automated chromatographic data system (PF Nelson 2600; Perkin-Elmer Corp., Cupertino, CA).

Results and Discussion

Effect of taxol on microtubule polymerization. In in vitro experiments, nanomolar concentrations of taxol (18-h exposure at 37°C) caused a dose-dependent increase in microtubule polymerization in VSMCs cultured on plastic (Fig. 1), cells that also exhibit the dedifferentiated phenotype (30). These results are striking in comparison with the "clinically relevant" micromolar (0.1–10) levels of taxol ordinarily required to cause microtubule bundling in various cultured cancer cell models (35–37). Interestingly, in phase I clinical trials for leukemia, the magnitude of the patient's clinical response to taxol was related to the in vitro sensitivity of leukemic blasts to form microtubule bundles (38, 39). Incubating VSMC with 10 nM taxol for 18 h also led to obvious cell rounding and polyploidy.

Effect of taxol on invasion and metalloproteinase secretion. Boyden chemotactic assays have been used previously to study in vitro tumor cell invasion (31, 32, 35), and more recently VSMC invasion (19), of basement membrane. This approach was used to evaluate how taxol-induced microtubule polymerization would impair vital cell processes necessary for in vivo neointimal formation, measured as the in vitro ability of cultured VSMCs pretreated with different taxol concentrations (30 pM to 100 nM) to invade filters coated with reconstituted basement membrane proteins in a 4-h Boyden chamber assay (Fig. 2) (19). Using PDGF-BB as an attractant, taxol inhibited VSMC invasion with a half-maximal inhibitory concentration (IC_{50}) of 0.5 nM. Taxol caused essentially complete inhibition at 100 nM, and significant inhibition was observed at 30 pM, the lowest dose used. A chemotaxis assay (filter coated with fibronectin and collagen I but no basement membrane protein barrier) with PDGF-BB as the attractant was performed in an analogous fashion, yielding the identical outcome (data not shown). Whereas cellular invasion of the reconstituted basement membrane protein barrier requires the secretion of specific collagenases (metalloproteinases) by VSMCs (19), as well as by tumor cells in other models (40, 41), these collagenases are not required for VSMC chemotaxis (19). These results suggest that taxol, at least at nanomolar drug levels, inhibits VSMC invasion primarily via inhibition of locomotion and/or shape changes, rather than by inhibition of cellular secretion of collagenases. Indeed, gelatinase zymography from these Boyden invasion experiments confirms that the level of VSMC type IV collagenase secretion did not vary significantly over the taxol range of 30 pM to 100 nM, compared with control (Fig. 2 *inset*). Dedifferentiated VSMCs are ~ 100- to 1,000-fold more sensitive to taxol inhibition than prostate carcinoma cells in a comparable invasion assay (35), as predicted by their respective susceptibilities to taxol-induced microtubule polymerization.

Further support for the concept that microtubule stabilization and hyperpolymerization are the critical and sufficient fac-

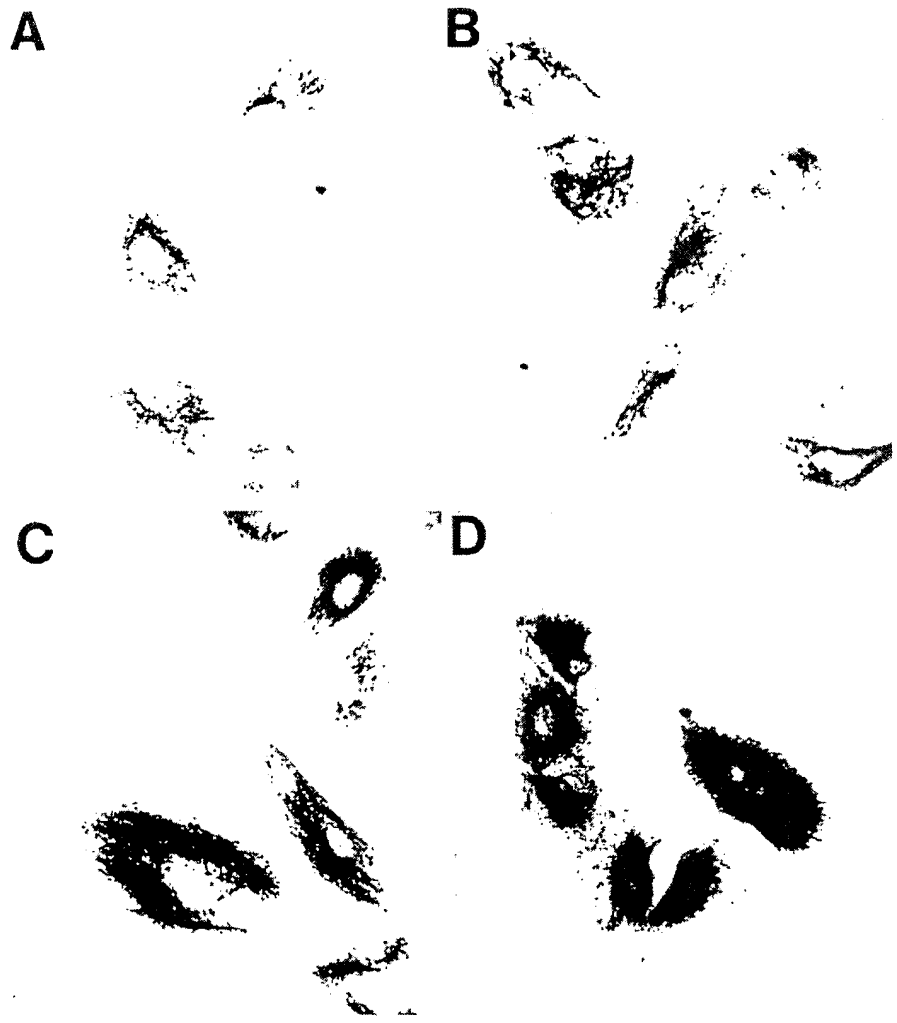

Figure 1. Indirect immunostaining of taxol-induced microtubule bundling in cultured vascular smooth muscle cells. After an 18-h taxol pretreatment in culture, cells were fixed, and polymerized β-tubulin was labeled with mouse anti-β-tubulin antibody. Secondary labeling was achieved with silver-enhanced, 1-nm gold-conjugated rabbit anti-mouse antibody. Representative light photomicrographs from (*A*) control, (*B*) 0.1 nM taxol-, (*C*) 1 nM taxol-, and (*D*) 10 nM taxol-treated VSMCs. ×450.

tors involved in taxol inhibition of PDGF-directed VSMC invasiveness comes from the results of additional experiments with ^2H_2O (heavy water), which enhances microtubule–tubulin polymerization via a mechanism distinct from that of taxol. A combination of the isotope and solvent effects of ^2H_2O (42) reversibly increases microtubule polymerization both by reducing the critical concentration for polymerization of $\alpha\beta$-tubulin heterodimers via enhanced tubulin hydrophobic interactions (43, 44) and by converting a population of unpolymerizable tubulin to the polymerizable form (45). Pretreating cultured VSMCs for 18 h with 25, 50, or 75% ^2H_2O caused dose-dependent microtubule hyperpolymerization similar to that observed with taxol (data not shown). This treatment likewise inhibited PDGF-mediated VSMC Boyden chamber invasion in a dose-dependent fashion, achieving half-maximal inhibition at ~ 25% ^2H_2O and nearly complete inhibition at 75% ^2H_2O (Fig. 3).

Effects of taxol on proliferation. In addition to cell recruitment and migration, the various growth-regulatory molecules elaborated after arterial injury, such as PDGF and basic fibroblast growth factor, are also implicated in mitogenesis and cellular proliferation (46–54). Taxol inhibited cultured VSMC [^{3}H]thymidine incorporation, an index of cell division, in a dose-dependent fashion, with an IC_{50} of 5.8 nM. Taxol caused essentially complete inhibition at 100 nM, and significant inhibition was resolvable at 1 nM (Fig. 2). That this inhibitory profile differs somewhat from that of invasion and chemotaxis, demonstrating 1 log–concentration unit lower sensitivity (IC_{50} 5.8±0.6 nM versus 0.5±0.1 nM, respectively, mean±SD [n = 3], $P < 0.001$) but with steeper concentration dependence (Hill coefficient 0.80±0.06 versus 0.39±0.04, respectively, mean±SD [n = 3], $P < 0.001$), likely arises because of the considerably different roles played by microtubules between these processes. ^2H_2O, which arrests mitosis in diverse animal and plant cell types (45, 55, 56) via impairment of the microtubule-organizing centers and thus of microtubule reorganization (57), similarly inhibited cultured VSMC proliferation and DNA synthesis (measured with the thymidine analogue BrdU incorporation into DNA) in a dose-dependent fashion (Fig. 3), consistent with the critical role of microtubule–tubulin dynamics in VSMC proliferation. The rightward-shifted ^2H_2O inhibitory profiles of proliferation versus invasion are qualitatively similar to those seen with taxol.

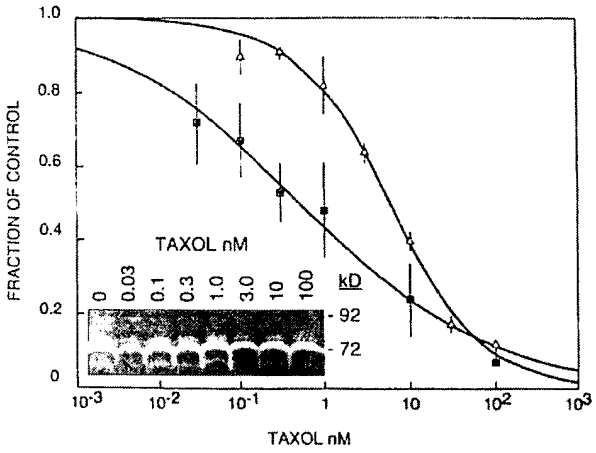

Figure 2. Taxol dose-dependent inhibition of PDGF-BB–directed VSMC chemoinvasion (■). 4-h chemoinvasion assays were performed using a modified Boyden chamber, seeded with 200,000 cells per well, and PDGF-BB (10 ng/ml) as the chemoattractant. Cells were pretreated for 18-h with taxol (concentrations 30 pM to 100 nM) in culture. All experiments were run in triplicate and were repeated three times on separate occasions using different VSMC preparations. Gelatinase zymograms were performed on the supernatants removed after the 4-h conclusion of the Boyden assays (*inset*). Gelatin-degrading metalloproteinases (including the 72-kD type IV collagenase) secreted into the medium by VSMCs were detected via SDS–PAGE; gels contained 0.1% gelatin and were stained with Coomassie Blue. The concave-downward aspect of the 72-kD band is an artifact arising from the presence of BSA in the original Boyden invasion assay and does not otherwise interfere with the 72-kD collagenase band. Taxol dose-dependent inhibition of VSMC DNA synthesis, as indexed by [^{3}H]thymidine incorporation (△). Each condition of these experiments was performed in triplicate. Error bars represent±SD values.

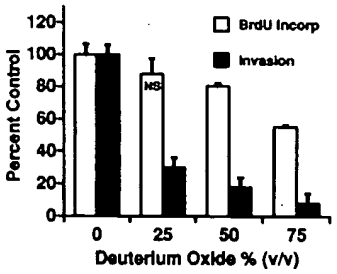

Figure 3. ^2H_2O dose-dependent inhibition of DNA synthesis and PDGF-BB–directed VSMC chemoinvasion. 4-h chemoinvasion assays were performed using a modified Boyden chamber, seeded with 200,000 cells per well, and PDGF-BB (10 ng/ml) as the chemoattractant. Cells were pretreated for 18 h with ^2H_2O (concentrations 0, 25, 50, and 75% [vol/vol] with H_2O) in culture (*solid bars*). All experiments were run in triplicate and were repeated three times on separate occasions using different VSMC preparations. Taxol dose-dependent inhibition of VSMC DNA synthesis, as indexed by the thymidine analogue BrdU incorporation (*open bars*). Each condition of these experiments was performed in triplicate. Error bars represent±SD values. NS, P not significant versus control. All other ^2H_2O treatment groups (both chemoinvasion and BrdU incorporation) were significantly different versus control ($P < 0.05$). Proportionate effects of ^2H_2O on chemoinvasion versus BrdU incorporation were significantly different ($P < 0.05$) at each ^2H_2O concentration (25, 50, and 75%).

In the rat, taxol at 2 mg/kg i.p., which resulted in peak plasma levels of 50–60 nM and trace levels (below the 20 nM HPLC quantitation limit) at 24 h, also inhibited in vivo medial VSMC proliferation (assessed by in situ BrdU labeling) by approximately half versus vehicle alone (11±3% versus 20±6%, respectively, mean±SD [n = 3 per group], $P < 0.05$) at day 2 after carotid balloon injury. These levels of inhibition are comparable to those achieved in in vitro conditions. These data are consistent with considerable experimental evidence that normal functional integrity of the microtubules is critical in the transmission of proliferative transmembrane signals from cell surface receptors to the nucleus (2–5, 58–61).

Effects of microtubule stabilization on neointimal formation. Thus, taxol significantly inhibits cultured VSMC in vitro invasion and proliferation through interference with microtubule function, disrupting locomotion and the facility to alter shape as well as growth factor–stimulated cell proliferation, at concentrations 10- to 1,000-fold lower than peak plasma concentrations achieved in treating human cancers (depending on the duration of the infusion schedule [3–24 h]) (62) and at pharmacological exposures substantially lower than those associated with minimal cytotoxicity in oncologic therapeutics (63, 64). This microtubule mechanism is supported by the analogous results of ^2H_2O experiments, which exert comparable microtubule effects via different underlying mechanisms. Furthermore, taxol, at doses achieving peak plasma levels approximately two orders of magnitude lower than those achieved in humans at doses used clinically to treat human malignancy, significantly inhibited VSMC proliferation measured near the time of its peak occurrence after vascular injury. To determine whether microtubule stabilization/hyperpolymerization could affect in vivo neointimal formation, we administered taxol in a rat model of arterial injury. Taxol administration in vivo (2 mg/kg i.p. for 5 d beginning 2 h after injury) inhibits the accumulation of neointimal smooth muscle cells in a rat carotid artery balloon catheter injury model, assessed at day 11, compared with vehicle alone (Fig. 4). Quantitative analysis of injured carotid segments showed that taxol treatment reduced the neointimal area by 70% compared with vehicle-treated animals ($P < 0.0002$) (Table I). Several of the taxol-treated animals showed virtually no discernable neointima (in the presence of denuded endothelium, proving injury), whereas all vehicle-treated animals demonstrated neointimal thickening. In separate experiments with this carotid injury model, experiments performed in rats ~ 95% equilibrated with 25% ^2H_2O demonstrate a ~ 40% reduction in neointimal area compared with normal H_2O-equilibrated control animals ($P < 0.05$) (Fig. 5; Table II), a degree of inhibition comparable to that observed at the same ^2H_2O level in the Boyden chamber assay.

Although taxol and ^2H_2O potentially affect multiple intracellular processes, the coincidence of their parallel effects on microtubules and on VSMC functionality at multiple levels suggests that microtubule stabilization is the likely mechanism of action, though alternative, unforeseen mechanisms may be responsible for these observed functional changes. Moreover, antimicrotubule agents and particularly those that stabilize and enhance microtubule polymerization, such as taxol, constitute some of the most potent antineoplastic strategies known, probably because of interference with a diverse array of vital cellular functions more critical to the transformed cell compared with the quiescent, differentiated cell. These experiments with taxol and ^2H_2O implicate microtubules in the control of the VSMC intracellular mechanisms necessary for promoting the multiple transformations involved in the development of the neointimal

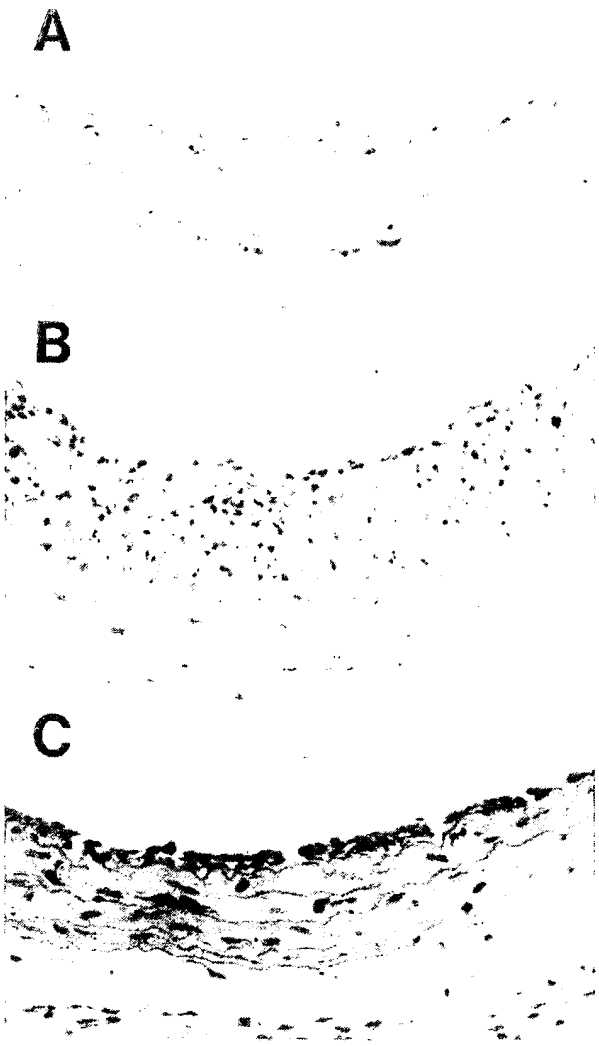

Figure 4. Taxol inhibits the accumulation of intimal smooth muscle cells 11 d after balloon catheter injury of rat carotid artery. The animals were treated with 2 mg/kg body weight taxol in vehicle (control animals were treated with vehicle alone) 2 h after the injury and daily for the next 4 d. Representative hematoxylin- and eosin-stained cross sections from (*A*) uninjured, (*B*) vehicle-treated, injured, and (*C*) taxol-treated, injured rat carotid arteries. ×240.

fibroproliferative lesion after arterial injury, making them particularly strategic targets to influence the outcome.

Based on the in vitro sensitivity of cultured human VSMCs to form taxol-induced microtubule polymerization, it may be possible to predict human clinical responses to these agents after arterial injury, as is true for certain leukemias (38, 39). Although the in vivo systemic taxol dose used in these experiments (yielding peak levels of 50–60 nM and trough plasma levels substantially lower than 20 nM) is significantly lower than that ordinarily achieved with doses and schedules commonly used to treat human cancers (approximately two orders of magnitude lower), even lower systemic dosing with sustained or even improved efficacy could be possible by combining a pretreatment regimen with the optimal treatment duration.

Table 1. Quantitation of the Effect of Taxol on the Accumulation of Intimal Smooth Muscle after Rat Left Common Carotid Artery Balloon Catheter Injury

Group	Intima	Media	I/M
	mm^2	mm^2	
Vehicle	0.09±0.01	0.14±0.01	0.66±.08
Taxol	0.03±0.01*	0.16±0.02‡	0.18±.04§

The image of each carotid artery 11 d after injury (8 taxol-treated and 10 vehicle-treated) was projected onto a digitizing board. The cross-sectional areas of the intima and the media were measured and are presented as the mean±SEM. I/M denotes the ratio of intimal to medial areas. * $P < 0.0002$, ‡ P = NS, § $P < 0.0001$, compared with vehicle, by unpaired t test.

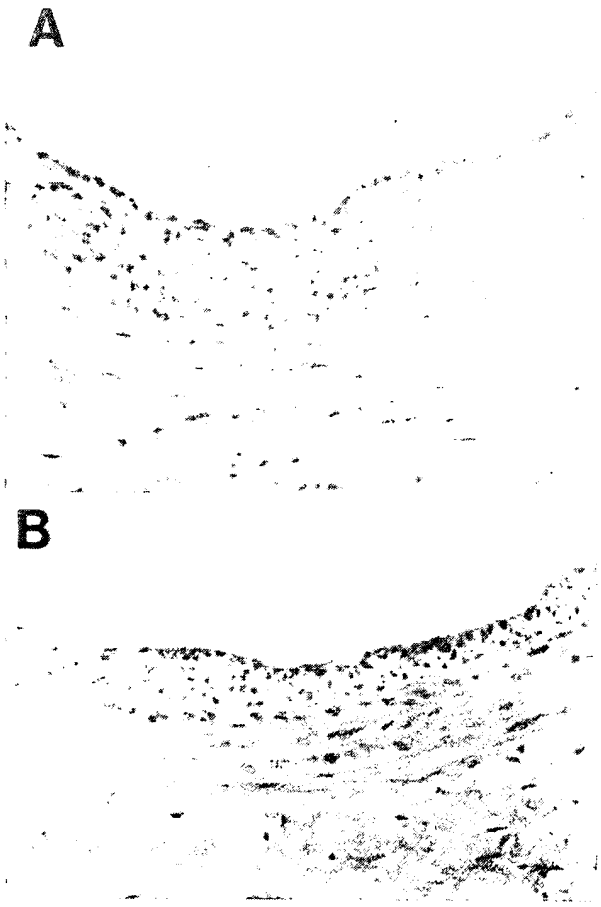

Figure 5. ^2H_2O inhibits the accumulation of intimal smooth muscle cells 11 d after balloon catheter injury of rat carotid artery. The animals were equilibrated with 25% ^2H_2O drinking water for 6 wk before carotid injury (a duration estimated to result in > 23% ^2H_2O replacement of body water) and until the carotid arteries were removed for study. Representative hematoxylin- and eosin-stained cross sections from (*A*) vehicle-treated, injured and (*B*) ^2H_2O-treated, injured rat carotid arteries. See Figure 4 *A* for a representative uninjured carotid section. ×240.

Table II. Quantitation of the Effect of ^2H_2O versus H_2O on the Accumulation of Intimal Smooth Muscle after Rat Left Common Carotid Artery Balloon Catheter Injury

Group	Intima	Media	I/M
	mm^2	mm^2	
H_2O	0.13±0.02	0.16±0.01	0.87±0.10
^2H_2O	0.08±0.01*	0.15±0.01‡	0.49±0.02§

The cross-sectional areas of the intima and the media of each carotid artery 11 d after injury (six ^2H_2O-equilibrated and six H_2O-equilibrated) were measured as in Table I, and the data are presented as the mean±SEM. I/M denotes the ratio of intimal to medial areas. * $P < 0.05$, ‡ P = NS, § $P < 0.005$, compared with control (H_2O), by unpaired t test.

Furthermore, a major goal of therapy after arterial injury is to inhibit the "activated" (i.e., after injury, dedifferentiated) VSMCs, or preferably to prevent activation, via temporary cytostatic mechanisms until the stimuli for growth and migration have abated (rather than causing cytotoxicity resulting in cell death). It is noteworthy that human taxol trials have demonstrated that hematopoietic effects (i.e., reductions in absolute neutrophil and white blood cell counts), the principal toxicity of taxol, begin to develop only when taxol plasma levels are maintained above an apparent threshold of 50–100 nM for durations beyond ~ 5 h (63, 64), conditions that exceed those used in the present experiments. Thus, the goal of short-term "restenosis-preventive" therapy with limited toxicity may be possible in humans after vascular surgical procedures if human and rat VSMCs are comparably sensitive to taxol.

Although the rat carotid artery injury model remains one of the most convenient and thoroughly investigated models for preliminary investigations into the mechanisms and treatment of restenosis, results from these studies are not necessarily predictive of therapeutic success in humans. The day 11 time point after carotid injury selected to assess neointimal formation in the present study, though predictive of outcome at day 14 (another typical experimental endpoint) in control rats in our laboratory (unpublished data), may not always be completely predictive of results at the longer term. Moreover, intimal hyperplasia, toward which taxol is apparently effective, is only one of several major mechanisms responsible for human restenosis. Additional phenomena, including vessel elastic recoil and wall remodeling, are important features of human restenosis that are probably incompletely addressed in the rat experimental model. Thus, further studies are needed in appropriate larger mammals.

Ultimately, local sustained-release delivery systems may offer the best solution to prevent human restenosis after angioplasty, enabling delivery of high local concentrations of drug and essentially eliminating problems of systemic toxicity. The development of taxol-impregnated biopolymer-coated stenting may offer a realistic approach to address these issues. These results offer the possibility of a chemotherapeutic approach to prevent clinical restenosis after angioplasty and other vascular surgical procedures, including bypass surgery, and possibly to attenuate cardiac transplantation–associated atherosclerosis.

Acknowledgments

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of the antithrombotic effects of a potent peptide-mimetic PAR-1 antagonist, RWJ-58259, by using two standard animal models. Since thrombin is implicated in the proliferative and inflammatory events associated with restenosis, we have also investigated the effects of RWJ-58259 in a rat model of vascular injury. Our results clearly suggest that a PAR-1 antagonist has the potential for therapeutic utility in restenosis following balloon angioplasty.

Experimental Procedures

Materials. RWJ-58259 was synthesized in our laboratories, purified by flash-column chromatography, and isolated as a dihydrochloride dihydrate (off-white powder). Details on the synthesis and isolation will be published separately. The structure of RWJ-58259 was confirmed by NMR spectroscopy and mass spectrometry; the purity was established by elemental microanalysis and reverse-phase high-pressure liquid chromatography.

Platelet Aggregation. Human platelet-rich plasma concentrate containing the anticoagulant acid-citrate dextrose (Biological Specialty Corp., Colmar, PA) was gel-filtered (Sepharose 2B, Amersham Pharmacia Biotech Inc., Piscataway, NJ) in Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.76 mM Na_2HPO_4, 5.5 mM dextrose, 5.0 mM Hepes, and 2 mg/ml bovine serum albumin, pH 7.4). Gel-filtered platelets were diluted with Tyrode's buffer (143,000 platelets/μl per well), compound solution in buffer, and 2 mM $CaCl_2$ in a 96-well microtiter plate. All fresh blood samples were obtained using sodium citrate (0.38% final concentration) as the anticoagulant. For platelet-rich plasma studies, human blood was obtained by venipuncture from healthy volunteers who were drug free for a minimum of 10 days. Guinea pigs (Hartley; Covance Inc., Denver, PA) or rats (Sprague-Dawley, Charles River, Raleigh, NC) were anesthetized and blood drawn via an intra-arterial catheter. Platelet-rich plasma was prepared by centrifugation at 200*g* for 10 min. Platelet-rich plasma aggregation was performed in the presence of 4 mM H-Gly-Pro-Arg-Pro-NH_2 to inhibit fibrin polymerization. Platelet aggregation was initiated by addition of an agonist shown to achieve 80% aggregation. The α-thrombin concentrations for gel-filtered platelet and platelet-rich plasma aggregation studies were 0.15 and 7.5 nM, respectively. The SFLLRN-NH_2 concentration used was 2 μM. The assay plate was gently mixed constantly. Aggregation was monitored at 0 and 5 min after agonist addition in a microplate reader by optical density at 650 nm (Molecular Devices, Sunnyvale, CA). Aggregation was calculated as the decrease in optical density between the two measurements. All samples were tested in duplicate wells on the same plate.

Cell Cultures. Human aortic smooth muscle cells and growth media were obtained from Cascade Biologics (Portland, OR). Rat aortic smooth muscle cells were obtained from Cell Applications (San Diego, CA) and were cultured as described (Owens et al., 1986).

Calcium Mobilization. Intracellular calcium mobilization was measured using a fluorescence technique. Rat aortic smooth muscle cells in 96-well microtiter plates were loaded with 5 μM fluo-3-AM (Molecular Probes, Eugene, OR) for 90 min. Plates were washed five times to remove unincorporated dye. Subsequent steps were performed using a fluorometric imaging plate reader (FLIPR, Molecular Devices). Test compounds were added and cells were monitored for 5 min to detect any inherent agonist activity. Thrombin (2 nM) was added and the fluorescence signal was recorded for 3 min. Net peak calcium, expressed in arbitrary fluorescence units, was measured automatically.

DNA Synthesis. Cell proliferation was measured by [^{14}C]thymidine incorporation. Rat aortic smooth muscle cells were plated on Cytostar scintillating plates (Amersham). After 4 days of growth, cells were depleted of serum for 4 days (Owens et al., 1986). Thrombin (0.8 nM) was added in fresh media and cells were incubated for 24 h. [^{14}C]Thymidine was added and incubation was continued for 24 h. [^{14}C]Thymidine incorporation was measured in a Wallac MicroBeta counter (Wallac, Gaithersburg, MD) without additional processing steps.

Interleukin-6 Assay. For measurement of interleukin-6 release, human aortic smooth muscle cells plated on 96-well microtiter plates were quiesced in Medium 231 (Cascade) containing 0.5% fetal bovine serum for 3 days. Thrombin (2 nM) was added in fresh serum-free Medium 231 and supernatants were collected after overnight incubation. Samples were analyzed by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

PCR Analysis for PAR-1 and PAR-4. Total RNA was isolated from guinea pig washed platelets using Trizol Reagent (Life Technologies, Grand Island, NY). For conversion of RNA to first-strand cDNA, samples were incubated with random primers in the presence or absence (minus RT for negative controls) of Superscript II reverse transcriptase (Life Technologies) according to the manufacturer's recommendations. PCR reactions were carried out on ca. 50 ng of cDNA, or equivalent amounts of RNA in the RT reactions, using the Advantage-GC cDNA polymerase mix (CLONTECH, Palo Alto, CA). Primers to generate and detect the respective guinea pig PAR amplicons were designed using the nucleic acid alignments of the known species for PAR-1 and PAR-3. However, numerous attempts to use this strategy to detect guinea pig PAR-4 were unsuccessful. Thus, the sequences used to amplify and detect the guinea pig PAR-4 PCR product were designed from the partial sequence analysis of the guinea pig PAR-4 gene (manuscript in preparation). The sense and antisense primers used for the amplification of PAR sequences were: PANP1-U, 5′-CATAAGCATTGACCGGTTCCTGGC-3′; PANP1-L, 5′-CAAAGCAGACGATGAAGATGCAGA-3′; PANP3-U, 5′-CAATGGCAACAACTGGGTATTTGG-3′; PANP3-L, 5′-AAAATCACAAGGATGAGGAG-3′; GPPANP4-U, 5′-TGGCCGTGGGGCTGCCGGCC-AATG-3′; and GPPANP4-L, 5′-GTCAACACAGCTGTTGAGGGTGCT-3′.

Reactions were conducted at a volume of 50 μl and at 25 cycles of 94°C for 30 s, 60.1°C for 30 s, and 68°C for 48 s for PAR-1; 20 cycles of 94°C for 30 s, 54.4°C for 30 s, 68°C for 56 s for PAR-3; and 28 cycles of 94°C for 30 s, 63.5°C for 30 s, and 68°C for 90 s for PAR-4. The products of each reaction (5.0 μl for PARs 1 and 3, and 50.0 μl for PAR-4) were electrophoresed through 2% agarose gels and transferred to Hybond N+ membranes (Amersham). The appropriate oligonucleotide primer probes, corresponding to nested sequences within the respective PAR PCR product, were digoxigenin-labeled, hybridized, and detected using the Genius nucleic acid detection system (Roche Molecular Biochemicals, Indianapolis, IN). The sequences used for these nested primer probes were: PANP1PP-L, 5′-CCAGAGTGCGCCAGGACAGGGACTGGATGGGGTACACCAC-3′ for PAR-1; PANP3PP-L#3, 5′-TCCTCACTTGCATGGGCATCAACCGCTACCTGGCCAC-3′ for PAR-3; and GPPANP4PP-L, 5′-CGGGCACGCAGGGGGTGCACCAGCGCCAGGTAGCGGTCCAGGCTGA-3′ for PAR-4.

Animal Models. All procedures involving the use of animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and the Animal Care and Use Committee, The R. W. Johnson Pharmaceutical Research Institute, Spring House, PA.

Ex Vivo Platelet Aggregation. RWJ-58259 was administered i.v. to anesthetized guinea pigs at the indicated doses as a 5 min infusion. Blood was withdrawn 5 min after dosing. Inhibition of thrombin or SFLLRN-induced platelet aggregation was assessed using platelet-rich plasma.

Guinea Pig Arteriovenous Shunt Thrombosis Model. Adult male guinea pigs (Hartley, 600–750 g) were anesthetized with a ketamine hydrochloride/xylazine hydrochloride solution i.m. The left jugular vein was cannulated (PE-50) for drug administration. The left carotid artery and right jugular vein were cannulated with silicon treated (Sigmacote, Sigma Chemical, St Louis, MO), saline-filled polyethylene tubing (PE-60) and connected with a 6-cm section of silicon-treated tubing (PE-190) to form an extracorporeal arteriovenous shunt. Shunt patency was monitored using a Doppler flow

system (model VF-1, Crystal Biotech Inc., Hopkinton, MA) and flow probe (1.0 mm, Titronics, Iowa City, IA) placed proximal to the shunt.

On completion of a 15-min postsurgical stabilization period, RWJ-58259 was administered intravenously as a loading-plus-maintenance infusion or directly into the shunt as a constant infusion. An occlusive thrombus was formed by the placement of a thrombogenic surface (#50 cotton thread, 6 cm in length) into the extracorporeal shunt. After 15 min exposure to flowing blood, the cotton thread was carefully removed and thrombus weight was calculated by subtracting the weight of the thread (3 mg) prior to placement from the total wet weight of the thread upon removal from the shunt. Arterial blood was withdrawn immediately at the conclusion of the study to assess ex vivo platelet function and coagulation.

Platelet count determinations were performed using a Sysmex K1000 differential cell counter (Sysmex Corporation, Kobe, Japan). Platelet-rich plasma aggregation induced by α-thrombin (35 nM) or SFLLRN-NH_2 (50 μM) was measured using an aggregation profiler (Bio/Data model PAP-4, Bio/Data Corp., Horsham, PA). Activated clotting time was determined using a whole-blood microcoagulation analyzer (Hemochron Jr., International Technidyne Corp., Edison, NJ). Template bleeding-time measurements were performed by the toenail-clip method, monitoring the time to clot formation.

RWJ-58259 was intravenously administered as a 5 mg/kg loading dose (over 5 or 10 min) with a subsequent 5 mg/kg maintenance infusion (over 20 min) for a total cumulative dose of 10 mg/kg. Inogatran (synthesized at the R. W. Johnson Pharmaceutical Research Institute) was administered as a 0.7 mg/kg loading dose (over 1 min) with a subsequent 0.3 mg/kg maintenance infusion (over 19 min) for a total cumulative dose of 1 mg/kg. Aspirin was administered at 100 mg/kg (over 2 min) and the shunt protocol was started 5 min later. This dose of aspirin was chosen based on previous studies whereby lower doses of aspirin had been ineffective in reducing thrombus weight. In a separate series of experiments, RWJ-58259 was administered directly into the shunt at a constant infusion of 0.1 or 0.3 mg/kg/min (over 20 min) for a total cumulative dose of 2 or 6 mg/kg, respectively. Inogatran was administered directly into the shunt at a constant infusion of 0.01 mg/kg/min (over 20 min) for a total cumulative dose of 0.2 mg/kg.

Intravascular Photoactivation Model. Male guinea pigs (Hartley, 375–700 g) were anesthetized with ketamine/xylazine (90/12 mg/kg, i.m.) and the right carotid artery gently isolated from the surrounding connective tissue. A 1-mm ultrasonic Doppler flow probe was secured around the artery proximal to the occlusion area and flow was continuously measured. Rose Bengal (Sigma), a photoactive dye, was dissolved in saline and infused i.v. at 20 mg/kg over 10 min. A green, heat-filtered xenon light source, positioned 0.5 cm from the artery to illuminate a 1-cm length of the vessel, was turned on 5 min before Rose Bengal infusion and remained on for 15 min. Arterial flow was monitored for a total of 30 min following the start of the Rose Bengal infusion. RWJ-58259 was administered at a total dose of 10 mg/kg, i.v., split into a 5 mg/kg infusion for 10 min prior to Rose Bengal and 5 mg/kg infusion starting after the conclusion of the Rose Bengal infusion for the remaining 20 min of the 30-min observation period. Recombinant hirudin (Hoechst Marion Roussel, Kansas City, MO) was infused at either 1 or 3 mg/kg i.v. for 10 min prior to the Rose Bengal infusion. RWJ-58259 was dissolved in 5% dextrose and r-hirudin was dissolved in saline. In a separate group of RWJ-58259-treated guinea pigs, not exposed to Rose Bengal or light, the animals were exsanguinated, platelet-rich plasma was prepared, and ex vivo platelet aggregation to α-thrombin and SFLLRN-NH_2 was measured.

Rat Restenosis Model. Vascular injury was induced by balloon-catheter inflation of the rat common carotid artery. A 2F embolectomy catheter was inserted via the external carotid into the left common carotid of male Sprague-Dawley rats (350–450 g) anesthetized with ketamine/xylazine (75/5 mg/kg, i.m.). The balloon tip was advanced to the aorta, inflated to 35 psi, and slowly withdrawn a total of three times. RWJ-58259 (1, 5, or 10 mg) was suspended in 150 μl of a polymer gel consisting of 50% capryolate and 50% glycolate and applied to the adventitia of the left common carotid. This polymer was shown not to affect the vascular injury response in this model. Perivascular treatment was used for these studies because RWJ-58259 is not orally active. Required intravenous infusion rates were not practical via minipump. This particular polymer has been successfully used for slow release of compounds. Since the material is absorbed slowly, we anticipated that RWJ-58259 would be released slowly over a period of time. Release kinetics were not performed for these studies. However, material tends to stay where placed and compound concentrations are expected to be high locally, likely resulting in significant levels reaching the luminal edge of the vessel. Fourteen days after injury, rats were anesthetized and perfusion-fixed with buffered formalin. Eight left carotid tissue sections (5 μm, 100 μm apart) were stained for elastin and used for morphometric analysis (Cheung et al., 1999). Medial and intimal area and thickness were measured using image analysis software. Percent stenosis was computed as intimal area as a percentage of the total area within the internal elastic lamina.

Data Analysis. All results are presented as mean ± S.E. Statistical analysis was performed either by the Student's t test or one-way analysis of variance where indicated. Mean values were considered statistically significant when $P < 0.05$.

Results

RWJ-58259 Is a Potent PAR-1 Antagonist. We recently described a series of indole-based peptide mimetics represented by RWJ-56110, which inhibits thrombin-induced PAR-1 activation in human platelets and vascular cells (Andrade-Gordon et al., 1999). Replacement of the indole template with an indazole template afforded an improved chemical series, represented by RWJ-58259 (Fig. 1). We selected this PAR-1 antagonist for animal studies because of its good potency, PAR-1 selectivity, and particularly, in vivo safety profile.

RWJ-58259 inhibited 0.15 nM α-thrombin and 2 μM SFLLRN-induced aggregation of human gel-filtered platelets with IC_{50} values of 0.37 ± 0.07 μM (n = 12) and 0.11 ± 0.01 μM (n = 9), respectively. The PAR-1 action of RWJ-58259 was verified by its failure to inhibit human gel-filtered platelet aggregation stimulated by either collagen or the thromboxane mimetic U46619. In addition, RWJ-58259 effectively inhibited human platelet-rich plasma aggregation induced by 7.5 nM α-thrombin (IC_{50}, 8.0 ± 2.0 μM, n = 3). The higher IC_{50} observed for RWJ-58259 in platelet-rich plasma studies most likely reflects both the elevated thrombin concentration required to activate platelets in plasma due to endogenous thrombin inhibitors as well as increased binding of RWJ-58259 to plasma proteins. At elevated concentrations of

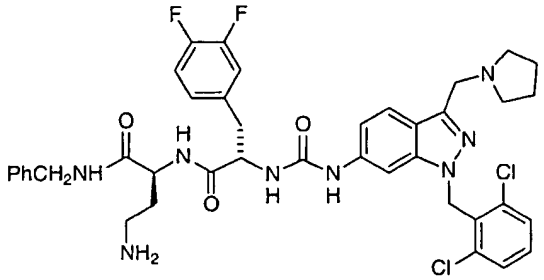

Fig. 1. Chemical structure of indazole-based peptide-mimetic RWJ-58259.

thrombin (e.g., 10–30 nM) with either human gel-filtered platelets or platelet-rich plasma, as observed previously for RWJ-56110 (Andrade-Gordon et al., 1999), RWJ-58259 became refractory in a thrombin dose-dependent manner, reflecting the dual PAR system on human platelets. The PAR-1 selectivity of RWJ-58259 was confirmed in the same, detailed fashion as described for RWJ-56110 (results not shown) (Andrade-Gordon et al., 1999).

In rat aortic smooth muscle cells, RWJ-58259 was found to inhibit α-thrombin-induced calcium mobilization (IC_{50} = 0.07 ± 0.01 μM, n = 4) and proliferation (IC_{50} = 2.3 ± 0.0 μM, n = 2). RWJ-58259 also blocked α-thrombin-induced interleukin-6 release from human aortic smooth muscle cells (IC_{50} = 3.6 ± 2.3 μM, n = 2). By contrast to human platelets, full antagonism of thrombin's action was observed in these vascular cells at high thrombin concentrations (e.g., 200 nM; results not shown). The ability of RWJ-58259 to inhibit signaling and function in smooth muscle cells, independent of thrombin concentration, is reflective of PAR-1 being the only thrombin-sensitive receptor on these cells (Andrade-Gordon et al., 1999).

Effects of RWJ-58259 on Guinea Pig Platelets. Guinea pig platelets have been widely used to test for PAR-1 action in platelet aggregation because they are responsive to the PAR-1-activating peptide SFLLRN-NH_2 (Connolly et al., 1994; Derian et al., 1995), which indicates the presence of functional PAR-1 on the cell surface. Since guinea pig platelets have a lot in common functionally with human platelets, we chose this small animal to explore PAR-1 antagonism in vivo. Our previous findings with the PAR-1 antagonist RWJ-56110 (Andrade-Gordon et al., 1999) confirmed the dual PAR activation system on human platelets. Given this background, we evaluated RWJ-58259 with guinea pig platelets for a similar mode of action. RWJ-58259 inhibited 7.5 nM α-thrombin-induced platelet-rich plasma aggregation with an IC_{50} of 7.4 ± 1.4 μM (n = 5), consistent with results from human platelet-rich plasma studies. Moreover, at a 10-fold higher concentration of α-thrombin, no inhibition was observed up to 100 μM RWJ-58259. In contrast, RWJ-58259 fully inhibited supramaximal concentrations of SFLLRN-NH_2 (100 μM)-mediated aggregation at a concentration of 10 μM. These results are indicative of another thrombin-sensitive receptor on guinea pig platelets in addition to PAR-1, as noted previously for human platelets. Because of the similarities between the in vitro behavior of RWJ-58259 in both human and guinea pig platelets, we considered this to be a suitable animal model for the investigation of PAR-1 physiology.

RWJ-58259 Inhibits ex Vivo Guinea Pig Platelet Aggregation. RWJ-58259 was first evaluated in a model of ex vivo platelet-rich plasma aggregation to determine the appropriate in vivo concentration ranges for further studies. RWJ-58259, administered to guinea pigs (0.3–3 mg/kg), inhibited α-thrombin-induced platelet-rich plasma aggregation in a concentration-dependent manner (Fig. 2). However, as α-thrombin concentrations were raised, RWJ-58259 became less effective, indicating that its ability to inhibit thrombin-mediated responses in vivo is dependent on the thrombin concentration. RWJ-58259-inhibited SFLLRN-induced aggregation under all conditions (results not shown). A dose of 10 mg/kg was chosen for further evaluations based on these results as well as pilot studies with earlier analogs including

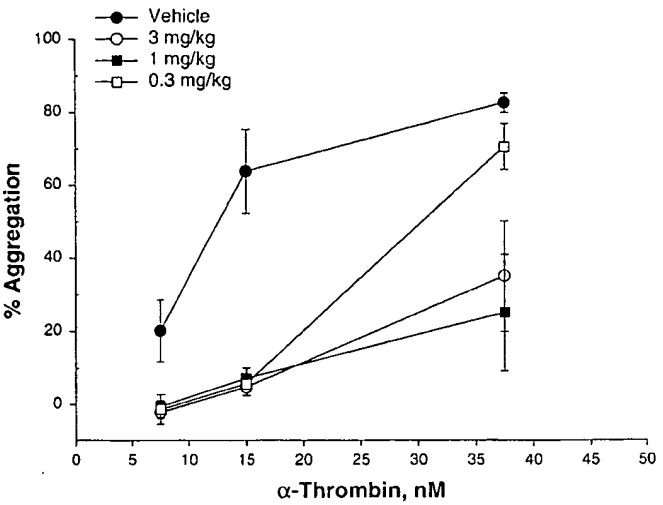

Fig. 2. Effects on ex vivo platelet-rich plasma aggregation after i.v. administration of RWJ-58259. Platelet-rich plasma aggregation in response to increasing concentrations of thrombin was inhibited after 0.3, 1, and 3 mg/kg of RWJ-58259.

RWJ-56110, which was ineffective at 6 mg/kg. This dose was the maximally tolerated intravenous dose for RWJ-58259.

Guinea Pig Arteriovenous Shunt Thrombosis Model. In this thrombosis model, a thrombus comprised of platelets, fibrin, and red blood cells forms on a section of cotton thread placed in an extracorporeal shunt between the carotid artery and jugular vein. Antithrombotic efficacy is indicated by decreases in the weight of thrombus accumulated during 15 min of exposure to flowing blood. Intravenous administration of RWJ-58259 (10 mg/kg) did not reduce thrombus weight (42 ± 4 mg, n = 2) when compared with a control group (43 ± 2 mg, n = 15) even though α-thrombin and SFLLRN-induced platelet-rich plasma aggregation were completely inhibited (Fig. 3A). The direct thrombin inhibitor inogatran (1 mg/kg, i.v.) or aspirin (100 mg/kg, i.v.) significantly decreased thrombus weight to 18 ± 3 mg (n = 6) and 16 ± 1 mg (n = 4), respectively. In a separate group of guinea pigs, RWJ-58259 was administered directly into the shunt just proximal to the thread in a protocol to maximize potential antithrombotic efficacy. An infusion rate of 0.1 mg/kg/min (2.0 mg/kg total dose) decreased thrombus weight slightly from a control of 35 ± 2 mg (n = 5) to 28 ± 4 mg (n = 4) (Fig. 3B). Increasing the infusion rate to 0.3 mg/kg/min (6.0 mg/kg total dose) further decreased thrombus weight to 24 ± 4 mg (n = 3). In these studies, the drug concentration (22 μM and 66 μM, respectively) was high enough to effectively inhibit α-thrombin and SFLLRN-induced platelet-rich plasma aggregation. Higher doses of RWJ-58259 could not be evaluated due to a combination of drug solubility and infusion volume. Bleeding times and activated clotting times were not changed. By comparison, administration of inogatran directly into the shunt at a rate of 0.01 mg/kg/min (0.2 mg/kg total dose) significantly decreased thrombus weight to 14 ± 2 mg (n = 3).

Guinea Pig Photoactivation Thrombosis Model. Intravascular photoactivation of the dye Rose Bengal with a green, heat-filtered xenon light results in endothelial damage that stimulates platelet adhesion to the vessel wall and generalized initiation of a platelet-rich thrombo-occlusive event. Antiplatelet agents and to a lesser extent, anticoagulants are

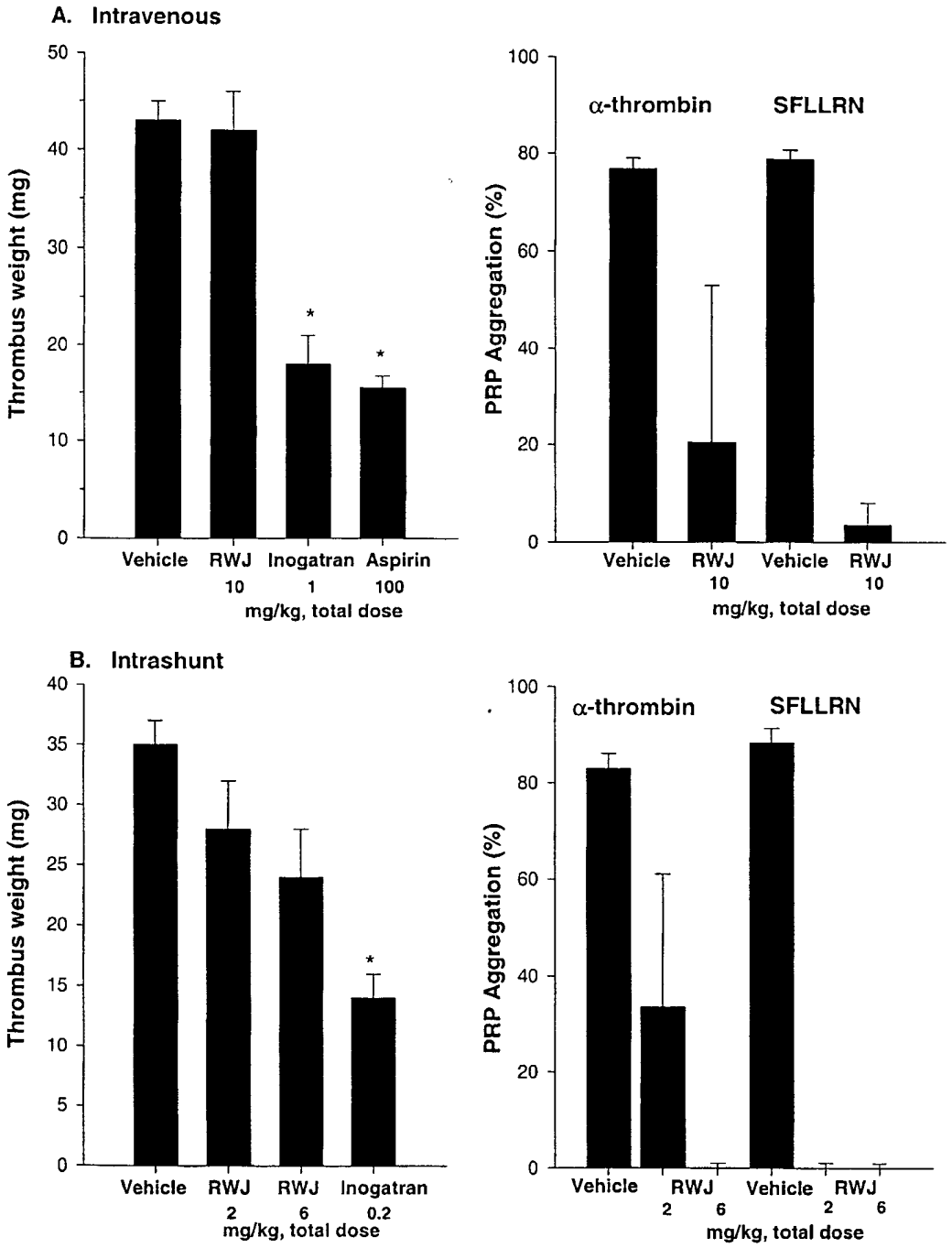

Fig. 3. Effect of RWJ-58259 (RWJ) in the guinea pig A-V shunt antithrombotic model. A, intravenous administration of RWJ-58259; B, intrashunt administration of RWJ-58259. Left panels, antithrombotic efficacy measured as reduction of thrombus weight compared with vehicle control; right panels, ex vivo platelet-rich plasma aggregation induced by 35 nM α-thrombin or 50 μM SFLLRN-NH_2. *$P < 0.05$ compared with vehicle treated by Student's paired t test.

effective in this model. Figure 4 (top panel) depicts carotid arterial perfusion and occlusion, as measured by Doppler flow, with each bar representing an individual animal. Initial occlusion times for the saline- and dextrose-treated animals averaged approximately 15 min. In all vehicle-treated animals except one, the arterial occlusion remained stable, whereas in the drug-treated groups the occlusion was unstable with intermittent flow observed over 30 min. At 1 mg/kg of the thrombin inhibitor r-hirudin, three of six treated animals were flowing at 30 min, and two of six did not experience occlusion. Two of eight RWJ-58259 treated animals were flowing at 30 min and one of eight did not experience occlusion. Total cumulative perfusion times (Fig. 4, middle panel) were significantly extended by r-hirudin at 1 and 3 mg/kg. RWJ-58259 at 10 mg/kg tended to increase perfusion times but this effect was not significant. RWJ-58259 significantly inhibited thrombin and SFLLRN-NH_2-induced platelet aggregation ex vivo (Fig. 4, bottom panel). Aggregation to low concentrations of α-thrombin (7–25 nM) was significantly inhibited, whereas aggregation at higher concentrations was much more variable and was determined not to be significantly different from that in the untreated animals. Aggregation to SFLLRN-NH_2 was completely inhibited by RWJ-58259 at all concentrations evaluated.

Guinea Pig Platelet PAR Profile. In vitro studies with our selective PAR-1 antagonist RWJ-58259 indicated the

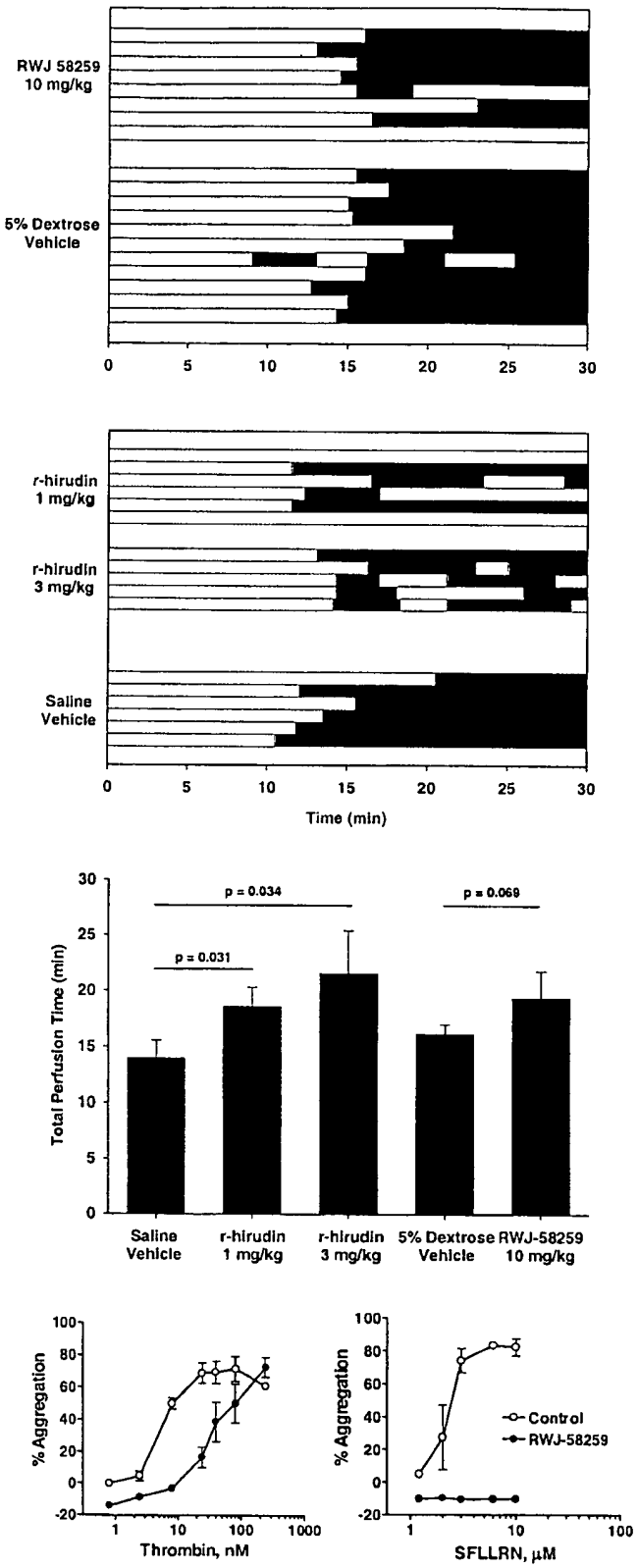

Fig. 4. Effect of RWJ-58259 in the guinea pig photoactivation model. Top panel, carotid arterial perfusion with each bar representing individual animals treated with vehicle, r-hirudin, or RWJ-58259; light shaded areas, flow; dark areas, no flow. Middle panel, average total, cumulative, perfusion time for each group. $*P < 0.05$ compared with vehicle control by Student's paired t test. Bottom panel, ex vivo aggregation of platelets prepared from control (○, $n = 3$) and RWJ-58259-treated (●, $n = 6$) guinea pigs.

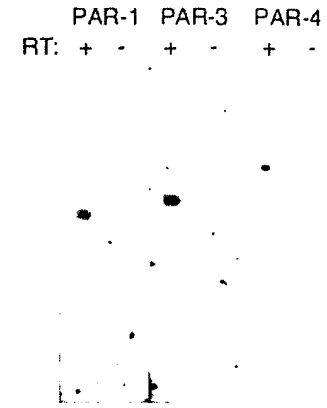

Fig. 5. PAR profile in guinea pig platelets. RT-PCR was used to examine the presence of PAR-1, PAR-3, and PAR-4 mRNA in guinea pig platelets. The predicted sizes of the amplicons, detected following hybridization with the appropriate nested primer probe, are as follows: PAR-1, 395 bp; PAR-3, 468 bp; and PAR-4, 746 bp.

presence of more than one thrombin receptor on both human and guinea pig platelets. Furthermore, results from the guinea pig in vivo thrombosis models suggested that another thrombin receptor, possibly PAR-4, plays a role in platelet-dependent thrombosis. Therefore, it was necessary to characterize the thrombin-receptor profile of guinea pig platelets. Although PAR-1 has been cloned from several species, only human and murine PAR-4 have been cloned and characterized (Kahn et al., 1998; Xu et al., 1998). Thus, we tested human PAR-1 (SFLLRN-NH_2)-, human PAR-4 (GYPGQV-NH_2)-, and murine PAR-4 (GYPGKF-NH_2)-activating peptides on human, rat, and guinea pig platelets. Whereas the human PAR-1 and PAR-4 peptides induced human platelet aggregation and the human and murine PAR-4 peptides induced rat platelet aggregation (no PAR-1 in rat platelets), only the PAR-1 peptide induced guinea pig platelet aggregation (not the PAR-4 peptides; results not shown). This outcome agrees with a recent communication by Nishikawa et al. (2000), in which washed guinea pig platelets do not respond to the murine PAR-4 peptide up to a concentration of 1 mM. To follow up on this observation, we examined the constitution of PARs in isolated guinea pig platelets by RT-PCR and were able to detect the mRNAs corresponding to PAR-1, PAR-3, and PAR-4 (Fig. 5). The apparent paradox of guinea pig platelets containing the message for PAR-4, but failing to respond to the human or murine PAR-4 peptides, was probed by isolating the guinea pig PAR-4 gene and characterizing the second exon.[1] Like the genomic organization of other so-characterized PARs, exon 2 of the guinea pig PAR-4 gene contains the coding sequences of the entire receptor, without the initiation codon and signal sequence. Interestingly, sequence analysis revealed that guinea pig PAR-4 contains the activation motif SFPGQA, which diverges from the motifs in human (GYPGQV) or murine (GYPGKF) PAR-4. We synthesized SFPGQA-NH_2 and found that it does induce the aggregation of guinea pig platelets with an EC_{50} of 131 μM. This result illustrates a notable flexibility in the evolution of the PAR-4 gene. In the final

[1] A. Darrow, C. Derian, M. Addo, and P. Andrade-Gordon, manuscript in preparation.

analysis, guinea pig platelets possess two functional thrombin-responsive systems, PAR-1 and PAR-3/PAR-4.

Effects of RWJ-58259 in a Rat Restenosis Model. Since α-thrombin-mediated vascular smooth muscle cell responses associated with vascular injury (inflammatory cytokine release and cell proliferation) were inhibited by RWJ-58259, this agent would be a good candidate to assess the role of PAR-1 in a rat balloon angioplasty model of vascular injury. Furthermore, since rat platelet aggregation stimulated by α-thrombin was not inhibited by RWJ-58259, confirming the lack of PAR-1 on these cells as well as the PAR-1 selectivity of RWJ-58259, this in vivo model would reflect effects directly on the vasculature. Perivascular treatment (1, 5, 10 mg) with RWJ-58259 produced dose-related reductions in intimal area and thickness, and a decrease in percent stenosis (Table 1), which became statistically significant at the 10 mg dose. Medial area and thickness were not changed, resulting in a significant reduction in the intimal to medial ratio. There was no evidence of an effect on remodeling. There was a trend toward increased lumen area at the 10 mg dose, but this was not significant. There was no significant difference in the vessel size among the treatment groups. An example of the effect of RWJ-58259 on vascular injury is shown in Fig. 6. Thus, there is a clear reduction in neointimal thickness in the section from a rat treated with RWJ-58259 compared with a section from a rat treated with vehicle. These results indicate that inhibition of thrombin-induced activation of PAR-1 in vivo can reduce the vascular injury response.

Discussion

The thrombin receptor PAR-1 has been implicated in a variety of cellular events mediated by thrombin, including those associated with thrombosis and vascular injury. In this report, we have demonstrated that PAR-1 is involved in the restenotic events associated with balloon angioplasty in rats by using a potent, selective PAR-1 antagonist, RWJ-58259. Furthermore, results with RWJ-58259 in two different guinea pig thrombosis models reveal that PAR-1 may partially mediate platelet-dependent thrombus generation; however, there are serious concerns about the suitability of this, and other, species for such antithrombotic studies.

Antithrombotic Effect of RWJ-58259. The presence of divergent thrombin-receptor profiles for platelets of different species was first recognized in studies employing the PAR-1 agonist peptide SFLLRN (Connolly et al., 1994; Derian et al., 1995). Platelets isolated from the blood of humans, primates, and guinea pigs, but not rabbits, rodents, and dogs, were responsive to SFLLRN, although all of the species responded to thrombin. Based on the species studies, we reasoned that the guinea pig would provide an appropriate small-animal model to assess platelet PAR-1-dependent responses associated with thrombosis. In both models evaluated, inhibition of thrombin's proteolytic activity resulted in significant antithrombotic effects, confirming a significant role for thrombin-mediated thrombus formation. Our results with RWJ-58259 revealed just a modest effect on thrombus formation in the two guinea pig models, raising the distinct possibility that PAR-1 is not a significant contributor to platelet thrombus formation. Our in vitro and in vivo platelet aggregation results with RWJ-58259 indicated that it is an effective antagonist of guinea pig PAR-1; however, its effectiveness was dependent on thrombin concentration. Complete antagonism of thrombin in vitro was achieved at low thrombin concentrations, but the effect diminished as the thrombin levels rose above 10 nM. Thus, we hypothesized that another thrombin-responsive receptor existed on guinea pig platelets.

Three thrombin receptors, PAR-1, PAR-3, and PAR-4, have been described and the PAR profiles of human and murine platelets have been reasonably well defined (Vu et al., 1991; Ishihara et al., 1997; Xu et al., 1998). On the basis of studies with PAR-3-deficient mice, it appears that a dual thrombin receptor system (PAR-3/PAR-4) exists on the platelets of wild-type mice (Kahn et al., 1998). However, human platelets do not express PAR-3 and thus PAR-1 was considered to be the only thrombin receptor on these cells. The discovery of human PAR-4 then suggested that human platelets do have a dual thrombin receptor system (PAR-1/PAR-4) (Xu et al., 1998). The presence of PAR-4 on human platelets is consistent with the loss of thrombin antagonist activity with our PAR-1 antagonists, RWJ-56110 and RWJ-58259 at elevated thrombin concentrations (Andrade-Gordon et al., 1999). Since the activity of RWJ-58259 was similar in isolated human and guinea pig platelets, we hypothesized that the results of our in vivo thrombosis models reflected a dual thrombin receptor system, PAR-1 and PAR-4, on guinea pig platelets.

Therefore, we sought to determine the PAR profile of guinea pig platelets, first by agonist peptide studies, then by RT-PCR. Surprisingly, our results indicate a triple PAR expression pattern with the presence of PAR-1, PAR-3, and PAR-4. This result raises important questions about the complex interactions of the different PARs during thrombus formation in different species and ultimately, the significance of PAR-4 activation in human thrombotic disease. The interaction of PAR-3 and PAR-4 was elegantly described by Nakanishi-Matsui et al. (2000), who demonstrated that PAR-3 serves as a cofactor for PAR-4, thereby increasing the thrombin sensitivity of PAR-4 by as much as 10-fold. The coordinated action of PAR-3/PAR-4 appears to mirror the action of

TABLE 1
Effects of adventitially administered RWJ-58259 on vascular injury in response to balloon angioplasty in the carotid artery of rats

Group	No.	Area, mm^2			Thickness, μM			Vessel Dimensions, mm^2	
		Intima	Media	% Stenosis	Intima	Media	I/M	Lumen	Vessel
Vehicle	10	0.129 ± 0.005	0.123 ± 0.006	44 ± 3	77 ± 5	58 ± 2	1.35 ± 0.09	0.231 ± 0.026	0.480 ± 0.028
1 mg	9	0.118 ± 0.010	0.118 ± 0.007	38 ± 2	68 ± 5	55 ± 2	1.24 ± 0.09	0.258 ± 0.019	0.482 ± 0.026
5 mg	9	0.110 ± 0.008	0.113 ± 0.007	36 ± 3	63 ± 4	53 ± 2	1.19 ± 0.09	0.239 ± 0.025	0.447 ± 0.035
10 mg	8	0.084 ± 0.007*	0.121 ± 0.006	26 ± 3*	45 ± 5*	54 ± 2	0.83 ± 0.10*	0.320 ± 0.031	0.507 ± 0.034

I/M, intima/media.
*$P < 0.05$, significantly different from vehicle by one-way analysis of variance.

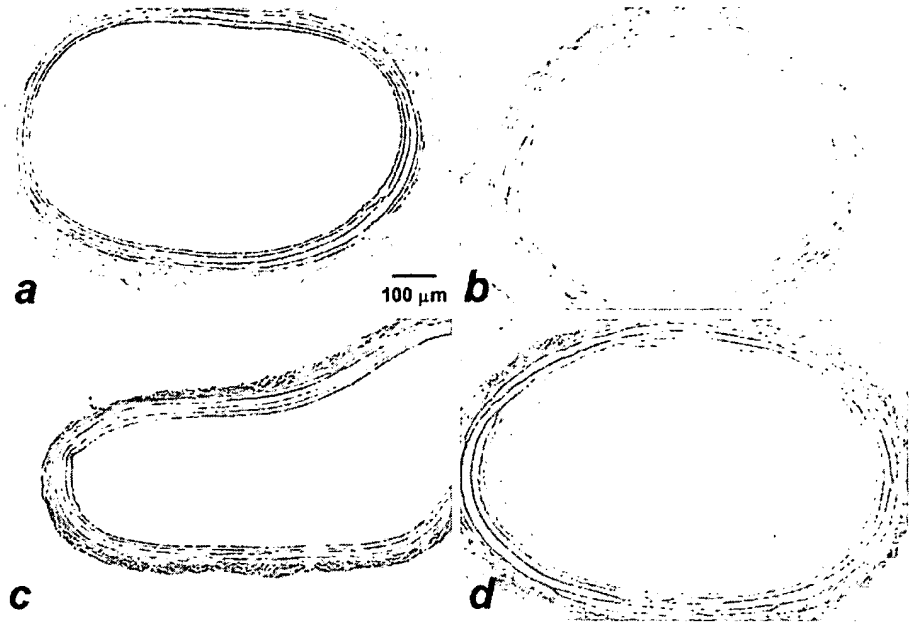

Fig. 6. Examples of rat carotid artery changes 14 days after vascular injury. A, uninjured, vehicle-treated; B, injured, vehicle-treated; C, uninjured, RWJ-58259-treated; and D, injured, RWJ-58259-treated. Scale bar, 100 μm.

PAR-1 with respect to thrombin sensitivity. The expression of all three PARs in guinea pig platelets suggests that two equally responsive thrombin systems, PAR-1 and PAR-3/PAR-4, exist there. The lack of significant antithrombotic activity for RWJ-58259 in the two guinea pig thrombosis models can be explained by the occurrence of thrombin-dependent platelet activation via PAR-3/PAR-4 during complete PAR-1 blockade. Our results indicate that guinea pigs may not be a suitable animal model for evaluating PAR-1 antagonists as potential antithrombotic drugs for humans.

The physiological role of this PAR redundancy may be a protective system to assure effective, rapid platelet aggregation during severe vascular injury, when concentrations of thrombin would be explosively elevated. Because of the complexity of multiple PARs, it has been difficult to dissect the contributions of individual PARs to the process of thrombosis, and this may have prevented the development of a PAR-1 antagonist as a potential therapeutic agent. The potential significance of PAR-4 activation in human clinical disease remains to be determined. Future studies in nonhuman primates, whose platelet PAR profile is similar to that of humans (unpublished observation), should provide a better means to evaluate the antithrombotic efficacy of selective PAR-1 antagonists.

Antirestenotic Action of RWJ-58259. Vascular injury associated with angioplasty procedures results from both thrombotic and restenotic components. While our results with RWJ-58259 in the thrombosis models did not conclusively determine the impact of PAR-1 antagonism on thrombotic processes, RWJ-58259 showed significant inhibition of neointimal thickening in the rat model of vascular injury, consistent with a direct effect on PAR-1-mediated vascular smooth muscle function. These results are highly significant, since rat platelets are fully responsive to thrombin through PAR-3/PAR-4 activation. Our results are consistent with a recent study that showed a reduced vascular injury response in rats treated with an antibody to PAR-1 (Takada et al., 1998). We have also found that the vascular injury response is reduced in mice deficient in PAR-1 compared with wild-type mice (Cheung et al., 1999).

PAR-1 is up-regulated in vascular smooth muscle cells in response to vascular injury in animal models (Wilcox et al., 1994; Cheung et al., 1999) and in human atherosclerotic coronary arteries (Nelken et al., 1992). This up-regulation is associated with proliferating cells. Thus, the effectiveness of PAR-1 antagonism in reducing vascular injury may be the result of inhibition of PAR-1-mediated vascular smooth muscle proliferation (McNamara et al., 1993). Consistent with this view, RWJ-58259 effectively inhibited thrombin-induced calcium mobilization and proliferation in rat aortic smooth muscle cells. Thrombin levels are also greatly increased at sites of vascular injury (Hatton et al., 1989; Harker et al., 1995). Although thrombin inhibitors have reduced vascular injury responses in several animal models (Heras et al., 1990; Barry et al., 1996; Gerdes et al., 1996), initial clinical trials have been unable to show the effectiveness of thrombin inhibition in vascular injury (Serruys et al., 1995; Burchenal et al., 1998). This observation may derive from inadequate treatment regimens. Alternatively, there may be some advantage to the specific blockade of PAR-1 as opposed to the inhibition of all of thrombin's many actions with a direct enzyme inhibitor.

In summary, we were unable to ascertain the antithrombotic potential of a PAR-1 antagonist in guinea pig models of thrombosis because of interference from the PAR-3/PAR-4 system present on guinea pig platelets. Thus, a determination of possible antithrombotic utility preclinically would require studies that surmount the species issue, such as through the use of primate models. However, our results with RWJ-58259 in rats indicate that selective antagonism of PAR-1 can significantly attenuate restenosis following balloon angioplasty. Accordingly, inhibition of PAR-1 may have therapeutic potential in human vascular injury.

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Blockade of the Thrombin Receptor Protease-Activated Receptor-1 with a Small-Molecule Antagonist Prevents Thrombus Formation and Vascular Occlusion in Nonhuman Primates

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ABSTRACT

Although it is well recognized that human platelet responses to α-thrombin are mediated by the protease-activated receptors PAR-1 and PAR-4, their role and relative importance in platelet-dependent human disease has not yet been elucidated. Because the expression profile of PARs in platelets from nonprimates differs from humans, we used cynomolgus monkeys to evaluate the role of PAR-1 in thrombosis. Based on reverse transcription-polymerase chain reaction, PAR expression in platelets from cynomolgus monkeys consisted primarily of PAR-1 and PAR-4, thereby mirroring the profile of human platelets. We probed the role of PAR-1 in a primate model of vascular injury-induced thrombosis with the selective PAR-1 antagonist (α*S*)-*N*-[(1*S*)-3-amino-1-[[(phenylmethyl)amino]carbonyl]propyl]-α-[[[[[1-(2,6-dichlorophenyl)methyl]-3-(1-pyrrolidinylmethyl)-1*H*-indazol-6-yl]amino]carbonyl]amino]-3,4-difluorobenzenepropanamide (RWJ-58259). After pretreatment with RWJ-58259 or vehicle, both carotid arteries of anesthetized monkeys were electrolytically injured and blood flow was monitored for 60 min. Time to occlusion was significantly extended after RWJ-58259 administration (27 ± 3 to 53 ± 8 min; $p < 0.048$). Vessels from three of the five treated animals remained patent. Ex vivo platelet aggregation measurements indicated complete PAR-1 inhibition, as well as an operational PAR-4 response. Immunohistochemical staining of mural thrombi with antibodies to the platelet marker CD61 and fibrinogen indicated that RWJ-58259 significantly reduced thrombus platelet deposition. Drug treatment had no effect on key hematological or coagulation parameters. Our results provide direct evidence that PAR-1 is the primary receptor that mediates α-thrombin's prothrombotic actions in primates and suggest that PAR-1 antagonists may have potential for the treatment of thrombotic disorders in humans.

Platelets play a major role in vascular occlusive diseases such as angina, myocardial infarction, and stroke, as a consequence of their inappropriate and sustained activation (Chesebro et al., 1992; White, 1999; Weksler, 2000). In these syndromes, the presence of lipid-laden atherosclerotic plaques within blood vessels fosters platelet-dependent thrombus formation, particularly after a vascular insult. The causative thrombi arise from platelet aggregates and fibrin deposits largely through the actions of the serine protease α-thrombin. Thus, the prevention of thrombin's prothrombotic effects by inhibiting its proteolytic activity or blocking its cellular actions should significantly attenuate thrombus formation and thereby counteract platelet-based vascular occlusion. Indeed, inhibitors of the enzymatic action of thrombin exert a therapeutically useful antithrombotic effect in humans (for reviews, see Gallo et al., 1999; Weitz and Buller, 2002). However, inhibition of thrombin's catalytic activity disrupts the normal hemostatic balance that is maintained by thrombin, and the continued generation of thrombin during antithrombin therapy can foster rethrombosis (Zoldhelyi et al., 1994; Kontny, 1997). Alternatively, the prevention of thrombin's action on platelets, without affecting thrombin's proteolytic actions, could provide a more focused and refined approach for regulating platelet-dependent thrombus formation.

Thrombin stimulates human platelets through the activation of two protease-activated G protein-coupled receptors, PAR-1 and PAR-4 (Vu et al., 1991; Kahn et al., 1998; Xu et

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ABBREVIATIONS: PAR, protease-activated receptor; PCR, polymerase chain reaction; PRP, platelet-rich plasma; RT, reverse transcriptase; RWJ-58259, (α*S*)-*N*-[(1*S*)-3-amino-1-[[(phenylmethyl)amino]carbonyl]propyl]-α-[[[[[1-(2,6-dichlorophenyl)methyl]-3-(1-pyrrolidinylmethyl)-1*H*-indazol-6-yl]amino]carbonyl]amino]-3,4-difluorobenzenepropanamide.

al., 1998). Since the discovery of PAR-1 and PAR-4 in human platelets, there has been a keen interest to dissect the specific involvement of each receptor in the process of platelet aggregation. In fact, transgenic mice deficient in these receptors have been obtained to probe for the contributions of different thrombin-sensitive PARs in platelet physiology (Connolly et al., 1996; Darrow et al., 1996; Nakanishi-Matsui et al., 2000; Sambrano et al., 2001). However, these studies have only led to indirect evidence to suggest that PAR-1 may be the primary activation mechanism for initiating human platelet activation. To examine the role of PAR-1 in arterial thrombosis, as well as to elucidate a potential role for PAR-4, we have developed selective PAR-1 antagonists, exemplified by RWJ-58259 (Andrade-Gordon et al., 2001; Zhang et al., 2001). Unfortunately, the evaluation of RWJ-58259 in small-animal models of thrombosis has been hindered by the species-dependent expression of PAR-1 in platelets (Connolly et al., 1994, 1996; Derian et al., 1995; Darrow et al., 1996). Because guinea pigs were identified as the only small animal possessing PAR-1 on their platelets, we studied the effect of PAR-1 inhibition with RWJ-58259 in two guinea pig models of arterial thrombosis (Andrade-Gordon et al., 2001). After our observation of marginal in vivo activity with RWJ-58259, we discovered that guinea pig platelets have a triple-PAR profile, which undoubtedly contributed to the lack of efficacy. Thus, there is a dearth of small-animal models for assessing the role of PAR-1 in thrombosis, due to the absence of platelet PAR-1 expression (Connolly et al., 1994; Derian et al., 1995) and/or species-dependent platelet PAR profiles (Kahn et al., 1998; Andrade-Gordon et al., 2001). We have now investigated the effect of the selective PAR-1 antagonist RWJ-58259 on thrombus formation subsequent to electrolytic injury in nonhuman primates. Our results demonstrate for the first time that selective in vivo blockade of PAR-1 attenuates thrombotic activity, even in the presence of platelet PAR-4, and suggest that PAR-1 antagonists have the potential for therapeutic utility in human thrombotic diseases.

Materials and Methods

Polymerase Chain Reaction (PCR) Analysis for PAR-1 and PAR-4. A PCR analysis was performed essentially as described previously (Andrade-Gordon et al., 2001) except that RNA samples were pretreated with RQ1 RNase-free DNase (Promega, Madison, WI) before cDNA synthesis. The sense (U) and antisense (L) primers for the amplification of PAR sequences were as follows: PANP1-U, 5′-CATAAGCATTGACCGGTTCCTGGC-3′; PANP1-L, 5′-CAAAGCAGACGATGAAGATGCAGA-3′; PANP3-U, 5′-CAATGGCAACAACTGGGTATTTGG-3′; PANP3-L, 5′-AAAATCACAAGGATGAGGAG-3′; PANP4-U, 5′-GCCAATGGGCTGGCGCTGTG-3′; PANP4-L, 5′-GCCAGGCAGATGAAGGCCGG-3′; β-actin PTP-U, 5′-AGGCCAACCGCGAGAAGATG-3′; and β-actin PTP-L, 5′-CTCGGCCGTGGTGGTGA AGC-3′. Reactions (50 μl) were subjected to 25 cycles of 94°C for 30 s/60.1°C for 30 s/68°C for 48 s for PAR-1; 30 cycles of 94°C for 30 s/54.4°C for 30 s/68°C for 56 s for PAR-3; 25 cycles of 94°C for 30 s/63.1°C for 30 s/68°C for 56 s for PAR-4; and 25 cycles of 94°C for 30 s/60.4°C for 30 s/68°C for 45 s for β-actin. The products of each reaction were electrophoresed through 2% agarose gels and transferred to Hybond N^+ membranes (Amersham Bioscience, Inc., Piscataway, NJ). The appropriate oligonucleotide primer probes, corresponding to nested sequences within the respective PAR PCR product, were digoxigenin-labeled, hybridized, and detected using the Genius nucleic acid detection system (Roche Diagnostics, Indianapolis, IN). The sequences used in this group of nested primer probes were as follows: PANP1PP-L, 5′-CAGAGTGCGCCAGGACAGGGACTGGATGGGGTACACCAC-3′ for PAR-1; PANP3PP-L, 5′-CCTGCTTCAGGATGACAAAGGGCAGCATGTATAAGAAAAC-3′ for PAR-3; PANP4PP-L, 5′-CCAGCAGCAACACTGAACCATACATGTGGCCATAGAG-3′ for PAR-4; and Actin PP-L, 5′-TGGGCACAGTGTGGGTGACCCCGTCACCGGAGTCCA TC AC-3′ for β-actin.

Carotid Injury Model of Thrombosis. We used an electrolytic injury-induced model of thrombosis in primates based on modifications to a similar model in dogs (Rote et al., 1994; Rebello et al., 1997). Ten cynomolgus monkeys of either sex, weighing 3 to 6 kg, were used in this study. All procedures involving the use of animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and the Animal Care and Use Committees at Charles River Laboratories and Johnson & Johnson Pharmaceutical Research and Development.

Animals were preanesthetized with ketamine hydrochloride, 10 mg/kg i.m., plus atropine sulfate, 0.04 mg/kg i.m., and immediately intubated and ventilated with a respirator. Anesthesia was maintained with isoflurane. End tidal CO_2 was monitored and maintained within individual physiological ranges. An intravenous catheter was placed in a peripheral vein for administration of lactated Ringer's solution at a rate of 5 to 10 ml/kg/h. Catheters were placed in a femoral vein and femoral artery for blood collection and monitoring blood pressure, respectively. Drug or vehicle was administered at a separate distant venous site. Both common carotid arteries were exposed and isolated via blunt dissection. A Doppler flow probe was placed around each carotid artery at a point proximal to the insertion of the intra-arterial electrode. Flow velocity was monitored continuously with a flowmeter (Transonic Systems, Inc., Ithaca, NY). A stenosis was created in each carotid using a vessel occluder. The degree of stenosis was adjusted so as to eliminate the hyperemic response after brief occlusion. Blood flow in the carotid arteries was monitored and recorded continuously throughout the observation periods using a physiological data acquisition and analysis system (Biopac, Santa Barbara, CA). An intravascular electrode (27-gauge needle) was inserted into each carotid at the point of stenosis. Electrolytic injury to the intimal surface of each carotid artery was induced by delivering continuous current (100 μA) from the positive pole of a model CCUI constant current unit (Grass/Astro-Med, West Warwick, RI). The negative terminal was connected to a distant subcutaneous site.

RWJ-58259 (Fig. 1; Zhang et al., 2001) was dissolved in a solution of 5% dextrose in water at a concentration to yield 1-ml/kg dose volumes for the bolus and infusion doses. The solution was administered intravenously as a bolus 5-min infusion at 3 mg/kg followed immediately by a continuous infusion for 65 min at 0.123 mg/kg/min. Control animals were treated with dose-equivalent volumes of 5% dextrose in water. Ten minutes after the start of treatment, anodal current was applied to the intra-arterial electrodes in both carotid arteries. Current was maintained for 60 min or until 15 min after the formation of a thrombotic occlusion in both vessels. Blood flow was monitored continuously before, during, and after induction of vessel injury. Occlusion was defined as flow of less than 1 ml/min. The

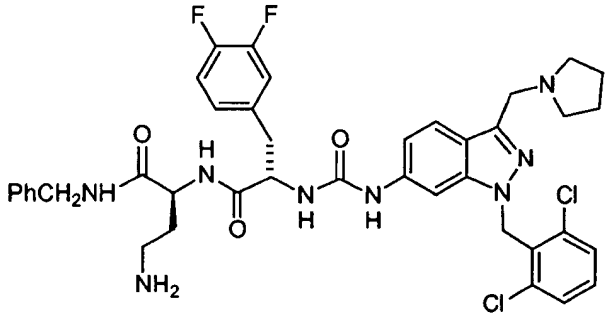

Fig. 1. Chemical structure of RWJ-58259.

incidence of occlusion and the time to occlusion (t) for the left and right carotids were recorded. If the arteries were patent at 60 min after electrical stimulation, a value of 60 min was used for statistical analysis. Blood samples were obtained before and after dosing for evaluation of platelet aggregation, hematology, clinical chemistry, coagulation profiles, and plasma drug concentration. At the termination of the experiment, each carotid was ligated proximal and distal to the point of injury, removed, and placed in fixative for histological analysis. At the end of the study, animals were euthanized with an overdose of sodium pentobarbital, performed in accordance with accepted American Veterinary Medical Association guidelines.

Platelet Aggregation and Coagulation Profiles. For in vitro aggregation studies, blood samples were obtained and platelet-rich plasma (PRP) was prepared as described previously (Derian et al., 1995). Platelets were pretreated with RWJ-58259 for 5 min before agonist stimulation. For ex vivo platelet aggregation studies, PRP prepared from treated and untreated animals was used as indicated above. The PRP aggregation assay was performed in the presence of 4 mM H-Gly-Pro-Arg-Pro-NH_2 to inhibit fibrin polymerization, specifically when α-thrombin was the agonist. The concentrations of α-thrombin used to stimulate PRP aggregation are consistent with the presence of endogenous antithrombins in plasma. The doses used were determined from preliminary studies to achieve maximal aggregation. The presence of H-Gly-Pro-Arg-Pro-NH_2 did not affect aggregation induced by peptide agonists. Aggregation was measured using an aggregation profiler (model PAP-4; Bio/Data, Horsham, PA).

Activated clotting time was determined using a Hemochron whole blood coagulation system model 801 (ITC, Edison, NJ). Prothrombin and activated partial thromboplastin times were determined using a hand-held monitor and cartridges (CoaguChek Plus system; Roche Diagnostics).

Histological Assessment of Carotid Thrombus. Formalin-fixed segments of injured carotids were embedded in paraffin and vessel cross sections were mounted and stained with hematoxylin and eosin to assess thrombus area. Thrombus composition was determined by single or double immunohistological staining of fibrin (anti-fibrin/fibrinogen; DAKO, Carpinteria, CA) and/or platelets (anti-CD61; DAKO) as described previously (D'Andrea et al., 2001). Fibrin-rich areas and platelet-rich areas were quantified by using ImagePro Image analysis software (Media Cybernetics, Silver Spring, MD). The total labeled area was expressed as a percentage of the total thrombus cross-sectional area. A representative section from each vessel was analyzed.

Data Analysis. All results are presented as mean ± S.E. Wilcoxon rank sum test was used for statistical analysis of occlusion times. One-way analysis of variance followed by Tukey's multiple comparison test was used for the platelet aggregation data. Student's t tests were used for the hematological and coagulation data and the histological platelet content data. Values were considered statistically significant when $p < 0.05$.

Results

Expression of PARs in Platelets of Cynomolgus Monkeys. We used the sensitive reverse transcriptase (RT)-PCR methodology to document the expression of PAR-1, PAR-3, and PAR-4 in platelets from cynomolgus monkeys. Total RNA was isolated from both monkey and human gel-filtered platelets, DNase treated, converted to cDNA by using RT, and subjected to PCR amplification. The sequences of the primers used to generate the respective PAR amplification products were designed from the conserved nucleic acid sequences of the known species PAR subtypes. The products of each PCR amplification were detected by Southern blot analysis using a nested oligonucleotide primer probe, corresponding to the appropriate PAR subtype. Transcripts for both PAR-1 and PAR-4 were detected in both human and monkey platelet RNA (Fig. 2). Under these conditions, the expression of PAR-3 was not detected in either human or monkey platelet RNA.

Arterial Thrombosis Injury Model. Based on the PCR profile of PARs, the cynomolgus monkey was deemed a suitable species to assess the importance of PAR-1 blockade on thrombotic occlusion. We used an experimental model of thrombosis involving electrolytic injury to the carotid arteries and subsequent thrombus formation, as assessed by absence of blood flow. The model was based on a modification of a similar model in dogs (Rote et al., 1994; Rebello et al., 1997). After intravenous infusion of RWJ-58259 or vehicle, the degree of vessel stenosis induced by electrolytic injury to each carotid artery independently was characterized by the incidence of occlusion and time to occlusion. Ten vessels from five vehicle-treated animals and nine vessels from five drug-treated animals were studied. One of the vessels in the RWJ-58259 group spontaneously occluded before treatment or electrolytic injury and was excluded from analysis. Application of anodal current to both carotid arteries of vehicle-treated animals led to total thrombotic occlusion in all vessels. The mean time to occlusion (t) was 27 ± 3 min (Fig. 3). RWJ-58259 significantly delayed or prevented occlusion in all of the vessels in five treated animals ($t > 30$ min) (Fig. 3). All carotid arteries from three of the five animals in the

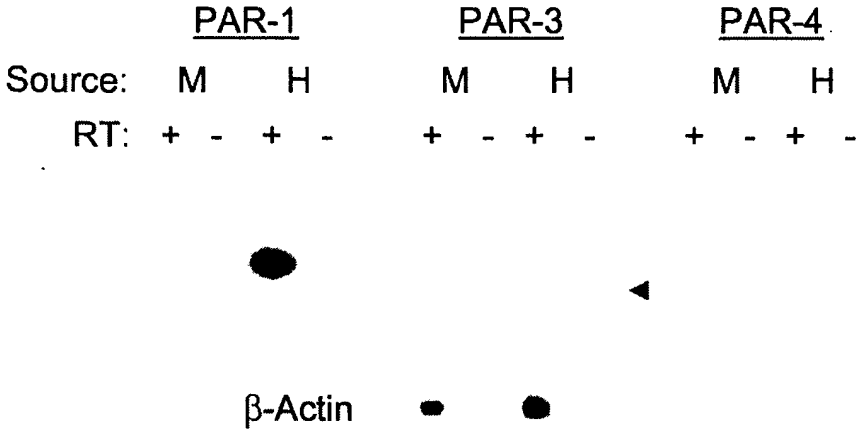

Fig. 2. Platelet PAR profile for cynomolgus monkeys. RNA extracted from monkey (M) and human (H) platelets was subjected to PCR analysis after treatment with (+) or without (−) RT. Samples were examined for β-actin to indicate that roughly equal amounts of RNA were analyzed. Platelets were positive for PAR-1 and PAR-4 by RT-PCR. The arrowhead denotes the expected location for PAR-3.

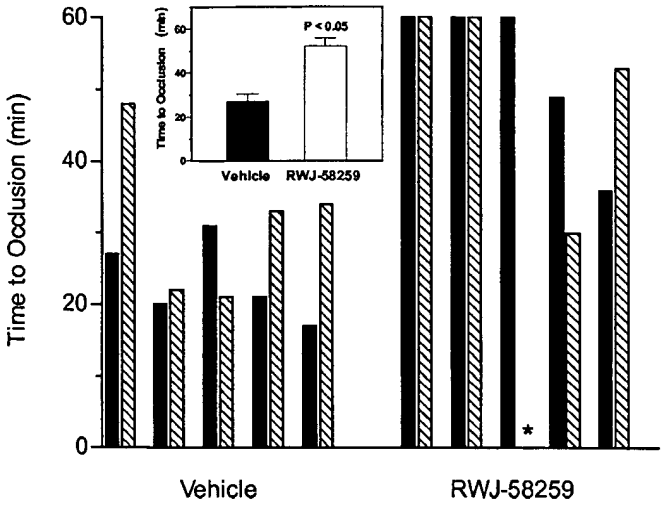

Fig. 3. Antithrombotic effect of RWJ-58259. Time-to-occlusion measurements are shown for individual animals after treatment with vehicle or RWJ-58259. The experiment was terminated at 60 min; thus, 60 min was used for calculations of mean time to occlusion in the insert. Solid columns represent the right carotid artery and hatched columns represent the left carotid artery. ⋆, indicates no data. Inset, mean ± S.E. from all vessels.

RWJ-58259 group remained patent during the 60-min observation period, despite continued application of current. Independent statistical comparisons between the right carotid artery ($p < 0.004$) and left carotid artery ($p < 0.048$) results from the vehicle and drug-treated groups indicated a significant effect of RWJ-58259 treatment on the prolonged time to occlusion.

In ex vivo platelet aggregation studies, aggregation in response to the PAR-1 agonist peptide SFLLRN-NH_2 and a low concentration of thrombin (38 nM) were completely inhibited both after the bolus dose and at the end of the observation period (Fig. 4). However, increasing concentrations of thrombin, which fully aggregated platelets in the absence of RWJ-58259, gradually overcame its inhibitory effect, consistent with its PAR-1 selectivity and the expression of PAR-4 on the platelets (Andrade-Gordon et al., 2001). Plasma levels of RWJ-58259 were 90 ± 15 μM at the end of the bolus infusion and 12 ± 3 μM ($n = 5$) at the end of the observation period. The latter concentration is consistent with inhibitory concentrations for PRP aggregation previously determined in human and guinea pig platelets (Andrade-Gordon et al., 2001). The PAR-1 selectivity of RWJ-58259 was confirmed in additional in vitro aggregation studies using monkey platelets. Agonist peptides for PAR-1 (SFLLRN) and PAR-4 (GYPGKF) stimulated full aggregation of monkey platelets (Fig. 5). RWJ-58259 completely inhibited SFLLRN-induced aggregation at a concentration of 1 μM, whereas doses as high as 20 μM had no effect on GYPGKF-induced aggregation, in agreement with its PAR-1 selectivity.

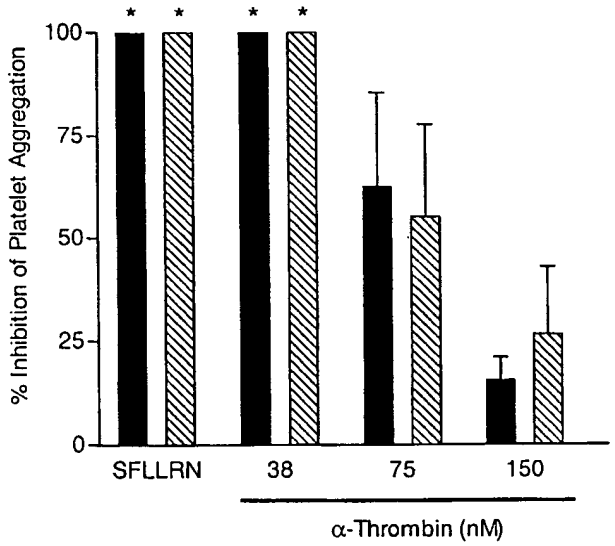

Fig. 4. Effect of RWJ-58259 administration on ex vivo platelet aggregation. Aggregation studies were performed with PRP samples obtained after the bolus dose (solid columns) and at the termination of the study (hatched columns). Platelet aggregation was induced by 30 μM SFLLRN-NH_2 or increasing concentrations of α-thrombin. Results are mean ± S.E. for four animals. ⋆, $p < 0.01$.

Thrombus Histology. Histological evaluation of the thrombi formed in the absence or presence of RWJ-58259 indicated significant differences in the composition of mural thrombi. Based on immunolabeling for the platelet marker CD61, there was a significant lack of platelet deposition in the existing thrombus of RWJ-58259-treated animals (Fig. 6, a and b). Immunolabeling of fibrinogen revealed the predominance of fibrinogen in the RWJ-58259 thrombi (Fig. 6, c and d). Representative sections from thrombi of vehicle and RWJ-58259-treated animals were subjected to image analysis to assess the overall composition of the thrombus. The platelet composition was reduced from 50 ± 3% ($n = 5$ vessels) in control-derived thrombi to 16 ± 8% ($n = 5$ vessels; $p < 0.01$) in RWJ-58259-derived thrombi. Thus, there seemed to be a switch from platelet-rich to platelet-poor thrombi in the presence of RWJ-58259

Hematology Profile. We compared the hematological profile as well as several coagulation parameters at baseline and at the termination of the study in both control and RWJ-58259-treated animals. There were no significant differences in red blood cell count, hematocrit, white blood cell count, or platelet counts between the two treatment groups at the start of the experiment and no significant changes in these parameters over the course of the experiment in either group (Table 1). Coagulation parameters, including activated clotting time, prothrombin time, and activated partial thromboplastin time were also within normal ranges and comparable in control and treated animals (Table 2). In addition, there were no significant changes in these parameters over the course of the experiment in either treatment group. Standard clinical chemistry assessments were also unaffected (data not shown).

Discussion

Thrombin plays a pivotal role in the development of arterial thrombi through activation of platelets and formation of fibrin (Fenton et al., 1993; Harker et al., 1995). The entrapment of thrombin within the fibrin meshwork of a thrombus provides a local elevation of thrombin protected from endogenous plasma-associated inhibitors such as antithrombin-III and heparin cofactor-II. The microenvironment of the growing thrombus presents a protected milieu for activation of platelets and other cell types such as leukocytes, which are known to reside within the thrombus. Direct thrombin inhibitors such as hirudin, bivalirudin, argatroban, and ximel-

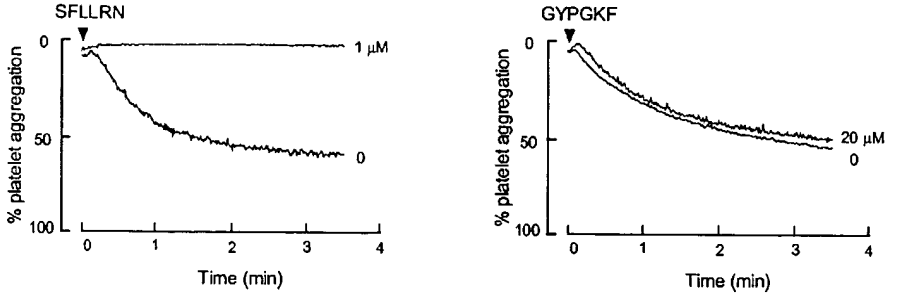

Fig. 5. RWJ-58259 selectively blocks PAR-1 in cynomolgus monkey platelets. PRP was stimulated with 10 μM SFLLRN (PAR-1-selective peptide) or 300 μM GYPGKF (PAR-4-selective peptide) to achieve maximal aggregation in the absence or presence of RWJ-58259, as described under *Materials and Methods*.

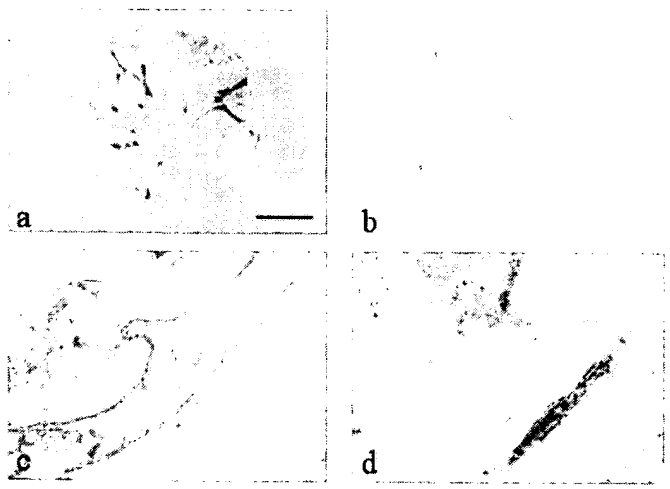

Fig. 6. Immunohistochemical staining of thrombus. a and c, representative thrombus from vehicle-treated animal; b and d, representative thrombus from RWJ-58259-treated animal. a and b, CD61 staining for platelets in thrombus represented by brown immunostaining. Scale bar, 250 μm. c and d, double immunohistochemical staining for platelets (CD61) and fibrin represented by brown and red staining, respectively. Scale bar, 100 μm.

agatran have progressed clinically on the basis that they can penetrate and inactivate clot-bound thrombin in contrast to heparins (Bates and Weitz, 1998; Mehta et al., 1998). However, the targeting of thrombin's enzymatic activity leads to both cellular and noncellular perturbations, which affect thrombin's procoagulant and anticoagulant activities. Indeed, clinical studies with these agents indicate positive outcomes in limiting arterial thrombosis with moderate changes in coagulation parameters such as activated partial prothromboplastin time (Badimon et al., 1994; Gallo et al., 1999; Weitz and Buller, 2002). Nonetheless, one of the most critical parameters still to be addressed is the potential for bleeding associated with this treatment modality. Thrombin's cellular actions, which are mediated through protease-activated receptors, play a critical role in arterial thrombosis. Thus, selectively blocking thrombin's cellular actions while sparing its normal hemostatic functions provides an opportunity to intervene more specifically during the formation and growth of a thrombus. The results reported herein demonstrate that nonhuman primates, exemplified by cynomolgus monkeys, offer a viable model to assess the potential efficacy of a PAR-1 antagonist for the prevention of arterial thrombosis in humans. We have determined that antagonism of PAR-1 in nonhuman primates provides significant protection against vascular injury-induced thrombosis.

The primate model of thrombosis used in this study is based on and adapted from a similar model in dogs in which electrolytic injury is used to induce thrombosis in a carotid artery (Rote et al., 1994; Toombs et al., 1995; Rebello et al., 1997). A number of antithrombotic and antiplatelet compounds have been studied and shown to be effective in this model, including direct and indirect thrombin inhibitors (Sudo and Lucchesi, 1996; Rebello et al., 1997) and platelet GPIIb/IIIa antagonists (Rote et al., 1994; Sudo et al., 1995). Thus, the model in the dog seems to be both platelet- and thrombin-dependent. Because it is likely that the mechanisms for thrombosis caused by electrolytic injury in dog and primate are similar, the antithrombotic activity of the thrombin receptor antagonist in our primate model is consistent with its antiplatelet and antithrombin mechanism of action. Another primate model of thrombosis involves mechanical injury, in which cyclic flow reductions occur as a result of cyclic formation of occlusive thrombi (Cook et al., 1995; Bellinger et al., 1998). In this model, both a GPIIb/IIIa antagonist and a direct thrombin inhibitor are effective in inhibiting cyclic flow reductions (Cook et al., 1995).

The therapeutic potential of a PAR-1 antagonist as an antithrombotic agent was first reported in a study with an antibody directed to the extracellular PAR-1 domain that binds thrombin's exosite region with high affinity (Cook et al., 1995). In this model of mechanical injury in African Green monkeys, the disruption of thrombin/PAR-1 binding effectively limited experimental thrombosis. More recently, two small peptides were reported to exert antithrombotic actions via PAR-1 antagonism. The heptapeptide AFLARAA inhibited arterial thrombosis in a rabbit model of electrolytic injury (Pakala et al., 2000), and the peptide RPPGF delayed coronary occlusion in a canine model using electrolytic injury (Hasan et al., 1996, 1999). In both cases, however, the mechanism of action of these peptides is unclear because PAR-1 is not expressed in either rabbit or canine platelets (Connolly et al., 1994; Derian et al., 1995). The development of a selective, small-molecule PAR-1 antagonist, such as RWJ-58259, which directly blocks the tethered ligand of PAR-1, provides a distinct interventional approach to thrombin-induced PAR-1 activation (Zhang et al., 2001).

We previously reported that RWJ-58259 does not effectively block arterial thrombosis in two separate models of thrombosis in guinea pigs (Andrade-Gordon et al., 2001). The lack of antithrombotic efficacy associated with RWJ-58259 in guinea pigs, one of the few species with PAR-1 on its platelets, relates to the triple PAR profile of these platelets, which could provide two fully functional thrombin-activating pathways, PAR-1 and PAR-3/PAR-4 (Nakanishi-Matsui et al., 2000; Andrade-Gordon et al., 2001). This curious outcome compels the use of a higher mammalian species that has PAR-1 on its platelets, in a manner consistent with human platelets. Our RT-PCR results indicate that PAR-1 and

TABLE 1

Effect of RWJ-58259 administration on the hematological profile of cynomolgus monkeys

Blood samples were obtained pre- and postvehicle or compound administration. Results are mean ± S.E. (n = 5 animals/treatment group).

	Vehicle-Treated		Drug-Treated	
	Baseline	65 min Post	Baseline	65 min Post
RBC ($10^6/\mu l$)	5.21 ± 0.79	4.23 ± 0.68	5.02 ± 0.40	4.28 ± 0.61
HCT (%)	36.62 ± 5.12	29.66 ± 4.58	34.82 ± 0.94	29.70 ± 2.74
PLT ($10^3/\mu l$)	329 ± 98	281 ± 113	311 ± 141	256 ± 106
WBC ($10^3/\mu l$)	8.14 ± 3.58	8.30 ± 2.88	7.3 ± 1.02	8.54 ± 1.46

TABLE 2

Effect of RWJ-58259 administration on coagulation parameters in cynomolgus monkeys

Coagulation profiles were run pre- and postvehicle or compound administration. Results are mean ± S.E. with the number of animals indicated in parentheses.

	Vehicle-Treated		RWJ-58259-Treated	
	Baseline	65 min Post	Baseline	65 min Post
ACT	97.5 ± 26.2 (2)	92.5 ± 34.6 (2)	107.3 ± 18.0 (3)	99.3 ± 32.5 (3)
PT	12.7 ± 1.5 (4)	14.1 ± 2.2 (4)	15.4 ± 6.4 (4)	15.1 ± 1.3 (3)
PTT	<18 (4)	<18 (3)	19.0 ± 3.0 (4)	19.5 ± 1.0 (4)

ACT, activated clotting time; PT, prothrombin time; PTT, activated partial thromboplastin time.

PAR-4 are expressed in the platelets of cynomolgus monkeys; however, no detectable PAR-3 was noted. These results are consistent with previous reports indicating relatively low expression of PAR-3 in human platelets based on both RT-PCR and protein expression (Schmidt et al., 1998; Kahn et al., 1999). The PAR-1 selectivity of RWJ-58259 demonstrated from our aggregation studies in cynomolgus monkeys coupled with our antithrombotic study indicates that blockade of PAR-1 is sufficient to protect against a significant vascular insult even when functional PAR-4 is present on platelets.

The physiological role of PAR-4 is not well understood because it is activated by relatively high concentrations of thrombin. In vitro studies with human platelets suggest its activation may serve as a secondary mechanism to ensure an antiplatelet response during vascular injury where thrombin concentrations are significantly elevated (Kahn et al., 1999). Although the complement of PAR receptors on murine and human platelets is distinct, some parallels can be drawn from studies using PAR-3- and PAR-4-deficient mice and the potential effects of a PAR-1 antagonist. Platelets isolated from PAR-3-deficient mice are less sensitive to thrombin stimulation compared with wild-type platelets, similar to the higher concentrations of thrombin needed to activate human platelet PAR-4 (Kahn et al., 1998). Hence, PAR-3 deficiency mirrors the potential effect a PAR-1 antagonist might have on human platelet responses to thrombin where PAR-4 signaling remains intact. In contrast, PAR-4 deficiency in murine platelets renders platelets totally unresponsive to thrombin consistent with its primary role as the thrombin-sensitive receptor and the cofactor role of PAR-3 (Sambrano et al., 2001). In vivo studies with PAR-4-deficient mice indicate that disabling of this primary thrombin-sensitive PAR provides protection from arteriolar thrombosis. This observation is consistent with blockade of PAR-1 on monkey or human platelets where PAR-1 is the primary thrombin-sensitive receptor and supports the concept that PAR-1 antagonism will provide sufficient antithrombotic efficacy after vascular injury. More recently, in vivo studies in PAR-3-deficient mice have shown a similar outcome (Weiss et al., 2002). The protection in these mice may again be attributed to the loss of platelet sensitivity to low nanomolar concentrations of thrombin, thus potentially shifting the response to one analogous to a human PAR-4 activation. Although direct associations cannot be made between the murine and primate studies, it is clear that thrombin's prothrombotic capacity is dependent on the complement and characteristics of the PAR profile.

There is increased bleeding in PAR-4-deficient mice, which indicates that PAR-4 plays a significant role in normal hemostasis. The lack of a secondary thrombin-activating pathway in murine platelets compared with human platelets, which have PAR-1 and PAR-4 acting independently, suggests that PAR-4 contributes to the maintenance of normal hemostasis in monkeys and humans. A potential role for PAR-4 in stabilizing platelet aggregates was revealed in studies with platelets from a patient with Hermansky-Pudlak Syndrome, a storage-pool deficiency (Covic et al., 2002). The mild bleeding diathesis observed in this patient was attributed to PAR-4 activation as a compensatory mechanism for ADP deficiency, resulting in relatively normal platelet aggregation. The ability to activate PAR-4 under conditions of exceptionally elevated thrombin concentrations thus allows for a fail-safe mechanism for maintaining hemostasis. Accordingly, we did not observe any effects on various coagulation parameters after blockade of PAR-1 with RWJ-58259. On the basis of our results, an avenue is now available to explore the role of platelet PAR-1 in human vascular occlusive disease. In this light, we propose that a selective PAR-1 antagonist has the potential for significant utility in cardiovascular therapeutics.

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REVIEW ARTICLE

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Novel Antithrombotic Drugs in Development

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Contents

Summary

Platelet activation plays a critical role in thromboembolic disorders, and aspirin remains a keystone in preventive strategies. This remarkable efficacy is rather unexpected, as aspirin selectively inhibits platelet aggregation mediated

through activation of the arachidonic-thromboxane pathway, but not platelet aggregation induced by adenosine diphosphate (ADP), collagen and low levels of thrombin. This apparent paradox has stimulated investigations on the effect of aspirin on eicosanoid-independent effects of aspirin on cellular signalling. It has also fostered the search for antiplatelet drugs inhibiting platelet aggregation at other levels than the acetylation of platelet cyclo-oxygenase, such as thromboxane synthase inhibitors and thromboxane receptor antagonists.

The final step of all platelet agonists is the functional expression of glycoprotein (GP) IIb/IIIa on the platelet surface, which ligates fibrinogen to link platelets together as part of the aggregation process. Agents that interact between GPIIb/IIIa and fibrinogen have been developed, which block GPIIb/IIIa, such as monoclonal antibodies to GPIIb/IIIa, and natural and synthetic peptides (disintegrins) containing the Arg-Gly-Asp (RGD) recognition sequence in fibrinogen and other adhesion macromolecules. Also, some non-peptide RGD mimetics have been developed which are orally active prodrugs. Stable analogues of prostacyclin, some of which are orally active, are also available.

Thrombin has a pivotal role in both platelet activation and fibrin generation. In addition to natural and recombinant human antithrombin III, direct antithrombin III–independent thrombin inhibitors have been developed as recombinant hirudin, hirulog, argatroban, boroarginine derivatives and single stranded DNA oligonucleotides (aptanes). Direct thrombin inhibitors do not affect thrombin generation and may leave some 'escaping' thrombin molecules unaffected. Inhibition of factor Xa can prevent thrombin generation and disrupt the thrombin feedback loop that amplifies thrombin production.

Three groups of substances need to be considered in the prevention of thromboembolic processes: substances which prevent platelet aggregation (platelet antiaggregating agents), agents which inhibit the coagulation process (anticoagulants) and substances which eliminate fibrinogen from the circulation (defibrinogenating agents).

Several strategies are currently being used to reduce platelet function: these are summarised in table I. In this review, inhibitors of prostaglandin (PG) synthase are not discussed as the effectiveness and noneffectiveness in terms of antithrombotic effects of aspirin and sulfinpyrazone, respectively, are well known. Dipyridamole, a drug which blocks adenosine monophosphate (AMP) breakdown by inhibiting phosphodiesterase in platelets[1] and which activates adenylase by a prostacyclin-mediated effect in the platelet membrane[2] has been widely used with meagre clinical support,[3] and cannot be considered to be a novel drug.

Numerous compounds which take part in the complex coagulation system, the inhibition of procoagulants as well as the stimulation of the natural endogenous inhibitors, can lead to an antithrombotic state. The mode of action and therapeutic properties of unfractionated and low molecular weight heparins[4-7] and of drugs inhibiting the synthesis of vitamin K–dependent coagulation factors (warfarin)[8] have been reviewed recently and are not discussed further.

Antithrombin III is the endogenous serine protease inhibitor (including thrombin) which can be concentrated from plasma or obtained by recombinant technology. Specific thrombin inhibitors became available for clinical evaluation [recombinant hirudin, hirulog, argatroban, RWJ-27755 (PPACK) and its derivatives]. Also, specific inhibitors of factor Xa have been purified from natural sources such as the soft tick leech (tick anticoagulant peptide; TAP) or Mexican leech (antistasin) or have been made synthetically (DX-90654). Protein C, another endogenous inhibitor, can be produced by recombinant technology, as has the lipoprotein-associated inhibitor produced by endothelial cells,

Table I. Strategies currently used to reduce platelet function

Inhibition of platelet enzyme prostaglandin synthase (e.g. aspirin, sulfinpyrazone, flurbiprofen, indobufen)
Inhibition of thromboxane synthase
Blockade of endoperoxide-thromboxane receptors or activators combined in one molecule
Inhibition of adenosine diphosphate–evoked signal transduction (e.g ticlopidine, clopidogrel)
Modulation of platelet adenylate or guanylate cyclase (e.g stable prostacycline analogues)
Interference with the function of the platelet glycoprotein Ib receptor (e.g. monoclonal antibodies) or the IIb/IIIa receptor (e.g. monoclonal antibodies, natural antagonists, synthetic peptides containing the RGD sequence, or nonpeptide inhibitors)
Peptides which bind to but do not inactivate the platelet receptor domain that interacts with thrombin

which is now termed tissue factor pathway inhibitor (TFPI).

Drugs which reduce blood viscosity such as the macropolymer dextrans have been reviewed;[9] defibrinogenating agents (ancrod, batroxobin) have been available for 30 years but have never become ingrained in medical practice and are omitted from this review.

1. Ticlopidine and Clopidogrel

These 2 thienopyridine derivatives can be considered as prodrugs since they are inactive *in vitro* but potent antiaggregating agents *in vivo*, indicating the importance of at least one active transient metabolite. The metabolic activation takes place in the liver as a portojugular shunt abolishes the antiaggregating effect. An alternative explanation is that the drug is acting at the megakaryocyte level.

Ticlopidine and its chemical analogue clopidogrel are noncompetitive but selective antagonists of adenosine diphosphate (ADP)-induced platelet aggregation. Since the 2 compounds are chemically related, their mechanism of action is considered similar. *Ex vivo* studies indicate that the antiaggregating effect is concentration-dependent; the rate of recovery is linked to platelet survival, suggesting a permanent effect on platelets.[10,11]

The mechanism of action of the 2 compounds is believed to be the inhibition of ADP-mediated direct and indirect actions on platelet aggregation. Both reduce responses to other agonists which require feedback amplification by ADP released from internal storage sites during granule secretion.[11,14] The binding of fibrinogen to glycoprotein IIb/IIIa (GPIIb/IIIa) complex, triggered by ADP, is dramatically inhibited; this inhibition is not due to a direct modification of the glycoprotein complex.[15] Ticlopidine and clopidogrel have no effect on phospholipase A activity or thromboxane A_2 and prostacyclin synthesis. They have no direct effect on either cyclic AMP (cAMP)-phosphodiesterase or adenylate cyclase. It has also been shown that ticlopidine potentiates the inhibition of platelet function and cAMP accumulation by the epoprostenol (prostacyclin; PGI_2)-mimetic iloprost. It was shown that ticlopidine increases the number of PGI_2 receptors in normocholesterolaemic or hypercholesterolaemic rabbits, while the number of thromboxane receptors remains unchanged.[16]

Clopidogrel is approximately 40 times as active as ticlopidine in inhibiting ADP-induced platelet aggregation in animal models, but about 6 times as potent as ticlopidine in tests of inhibition of ADP-induced aggregation of human platelets. Ticlopidine facilitates the disaggregation of thrombin-induced platelet aggregates most probably because it inhibits the effects of ADP on platelets.[17]

The 2 drugs have no effect on platelet-collagen adhesion which seems not to be affected by ADP, in contrast to platelet-platelet binding.[18] Neither has an effect on coagulation and fibrinolysis, but they may reduce myointimal thickening of the arterial wall, presumably by the inhibition of platelet aggregation and the subsequent release of platelet-derived growth factor.

Ticlopidine and clopidogrel prolong the bleeding time, with a maximal effect seen after 5 to 6 days of repeated oral administration compared with a lag time of 3 to 5 days before maximal inhibition of platelet aggregation is seen.[19,20] Clopidogrel appears to act faster than ticlopidine.

Ticlopidine and clopidogrel have been tested in several animal models of platelet-dependent arterial or venous thrombosis and found to be more

effective than sulfinpyrazone, dipyridamole and aspirin (reviewed by Saltiel and Ward,[10] Panak et al.[20] and McTavish et al.[21]). Other effects are a reduction in fibrinogen levels and blood viscosity and normalisation of decreased erythrocyte deformability.[10]

The effectiveness of ticlopidine has been convincingly demonstrated in patients at high risk of arterial thromboembolic events, i.e. those with transient ischaemic cerebral attacks and stroke, peripheral arterial disease or ischaemic heart disease.[21,22] A large trial in more than 3000 patients has shown that ticlopidine has a more pronounced effect on death from all causes or nonfatal stroke than aspirin.[23,24]

The most common adverse effects associated with ticlopidine are gastrointestinal symptoms: diarrhoea is the most frequently reported, affecting about 20% of treated patients. Other effects are skin reactions (urticaria, pruritus, erythema), haemorrhagic disorders (epistaxis, ecchymoses, menorrhagia). These effects are generally not severe and resolve after discontinuation of ticlopidine. The most potentially serious problem is bone marrow depression (leucopenia, thrombocytopenia, pancytopenia); close monitoring is therefore essential for at least the first 12 weeks of ticlopidine therapy.[21] Ticlopidine has also been associated with an increase in total cholesterol levels by 9%.[23]

Clopidogrel was developed because this compound was not toxic to bone marrow pluripotent stem cells in the mouse (Till and McCullogh test). Also, in phase II studies adverse events with clopidogrel were proportionally less frequent than with ticlopidine and not related to the dose.

A phase III study with clopidogrel (CAPRIE, Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events) is currently being conducted to assess the relative efficacy, safety and tolerability of clopidogrel and aspirin in reducing the incidence of the composite outcome of ischaemic stroke, myocardial infarction (MI) or vascular death among patients who have survived a recent ischaemic stroke or MI, or who have symptomatic atherosclerotic peripheral arterial disease. The study is a randomised, stratified, multicentre, double-blind, parallel-group design. Eligible patients are randomly assigned to receive either clopidogrel 75 mg/day or aspirin 325 mg/day for a maximum of 3 years. The number of patients enrolled is over 19 000, with at least 5000 patients in each of 3 clinical subgroups: recent ischaemic stroke, recent MI and peripheral arterial disease. Safety is assessed by frequent clinical and laboratory monitoring, especially in the first 3 months after study drug administration. The External Safety and Efficacy Monitoring Committee (ESEMC) performs unblinded quarterly reviews of all available CAPRIE data and has indicated that the study can continue to its completion.

2. Glycoprotein IIb/IIIa Antagonists

The heterodimer GPIIb/IIIa complex is a member of the integrin superfamily of receptors which mediates such apparently diverse phenomena as platelet aggregation, platelet adhesion to collagen, fibroblast adhesion to fibronectin and vitronectin, and leucocyte binding to endothelial cells and matrix proteins.

Expression of the GPIIb/IIIa complex and its binding to fibrinogen is the final common pathway for all platelet agonists. Other plasma adhesive proteins which may mediate platelet aggregation through binding to the GPIIb/IIIa complex are von Willebrand factor, particularly under conditions of high shear stress, and fibronectin.[25] The amino acid sequence 95-97 (Arg-Gly-Asp or RGD) and 572-575 (Arg-Gly-Asp-Ser or RGDS) of fibrinogen, von Willebrand factor and fibronectin binds to the activated GPIIb/IIIa receptor. These sequences occur in each alpha chain of fibrinogen. Another distinct amino acid sequence, a dodecapeptide, present in the carboxy terminal of each fibrinogen gamma chain, also binds to GPIIb/IIIa.[26,27]

The first platelet GPIIb/IIIa antagonists to be developed were murine *monoclonal antibodies*.[28,29] *In vitro*, these antibodies completely inhibited platelet aggregation and, in animal models

of angioplasty injury and thrombolysis, prevented thrombosis and augmented the activity of thrombolytic agents.[30,31] Because of concerns about their immunogenicity, the derivative product, chimaeric monoclonal 7E3 Fab (abciximab), was created via genetic recombination. This new hybrid molecule consists of the mouse-derived variable regions from the original molecule linked to the constant region derived from human immunoglobulin IgG.

Data from a dose-escalation study[32] and a pilot therapeutic trial[33] suggested an abciximab dosage regimen which was evaluated in high-risk patients undergoing percutaneous transluminal coronary angioplasty (PTCA).[34,35] Compared with placebo, an abciximab bolus of 0.25 mg/kg followed by an infusion of 10 μg/min for 12 hours in 708 patients resulted in a 35% reduction in the rate of the primary end-points (death, nonfatal MI, unplanned surgical revascularisation, unplanned repeat PTCA, stent or balloon pump for refractory ischaemia). However, bleeding episodes and transfusions were more frequent in abciximab recipients.[34] At 6 months, the absolute difference in patients with a major ischaemic event or elective revascularisation was 8.1% between the placebo and abciximab bolus plus infusion groups.[35]

The efficacy of abciximab has also been tested in patients with unstable angina unresponsive to heparin, aspirin and nitrate therapy who were undergoing PTCA. This placebo-controlled pilot study in 60 patients showed a substantial reduction ($p = 0.052$) in major clinical events (death, MI, urgent invasive intervention for recurrent ischaemia).[36] The large multicentre CAPTURE trial in 1400 patients with refractory unstable angina is presently being conducted, as a follow-up to this pilot study.

A number of *naturally occurring cysteine-rich single chain snake venom polypeptides* (disintegrins) were found to prevent binding of all adhesive proteins to GPIIb/IIIa. The antithrombotic potential of the disintegrins is dose-dependent. Some of these agents are kristin (*Agkristrodon rhodostoma*[37]), bitistasin (*Bitis arietans*[38]), applaggin (*Agkristodon piscivorus piscivorus*[39]), echistatin (*Echis carinatus*) and trigamin (*Trimeresurus gramineus*[40-43]). Barbourin, a peptide isolated from *Sistrurus m. barbouri*, has Arg substituted fro Lys, and affects the binding to GPIIb/IIIa on platelets only[44] while the other snake peptides block also GPIIb/IIIa receptors on other cells. These nonenzymatic native peptides have a low potency and short half-life ($t_{1/2}$), which diminishes their therapeutic value. It could be argued that the short $t_{1/2}$ is an advantage in case of bleeding. GPIIb/IIIA antagonists do not inhibit platelet release reaction or thromboxane A_2 (TXA_2) synthesis.

A series of *synthetic peptides containing the RGD or analogous sequence* which compete with fibrinogen for the GPIIb/IIIa binding site have been designed. Several of these peptidomimetics[44-49] and nonpeptidomimetics[50] are now available for clinical studies.

The *synthetic antiplatelet peptides*, particularly those in cyclic configuration, are potent antithrombotic agents when tested in platelet-mediated thrombosis in different experimental animals. While short cyclic synthetic peptides have a higher potency they also lack specificity for GPIIb/IIIa and recognise receptors on several integrins. The most potent compounds, at doses required for effective inhibition of *in vivo* thrombus formation, also induce a haemorrhagic tendency, as witnessed by the marked prolongation of the bleeding time.[51] Structure-activity studies have revealed a partial dissociation between the inhibition of *ex vivo* platelet aggregation and bleeding time prolongation and suggest that it might be possible to obtain GPIIb/IIIa antagonists with an optimised antithrombotic versus haemorrhagic ratio.[52]

The *nonpeptide inhibitors* of GPIIb/IIIa have obvious advantages compared with monoclonal antibodies as their effects are much shorter [3 hours for tirofiban (MK-383) versus 3 days for abciximab[53]] and have the potential to be active orally.

SC-5468A is a prodrug of a nonpeptide mimetic of the tetrapeptide RGDF. The active metabolite SC-54701A is a potent inhibitor of GPIIb/IIIa, and

exhibits specificity for this receptor with respect to other integrins.[54] More than 50% of the orally administered prodrug is absorbed in dogs and half that amount is converted to the active agent.[54] Platelet aggregation is completely inhibited for more than 8 hours after a single oral dose of 2.5 mg/kg. After intravenous administration, the elimination $t_{1/2}$ of the active moiety is 6.5 hours (4.7 ± 0.1 hours) in dogs and the total plasma clearance is 0.3 L/h/kg. The results of a dose-ranging study show that oral administration of the prodrug produces a dose-dependent inhibition of platelet aggregation which is maintained during a 14-day administration period in dogs, with no adverse effects.[55] At a dose inhibiting collagen-induced aggregation by 80%, bleeding time was increased 2.5-fold. Whether these results will be translated into less bleeding in a clinical situation is still to be demonstrated.

Fradafibran (BIBU 104XX) is the orally available prodrug of the fibrinogen receptor antagonist BIBU 52ZW. Escalating single and multiple oral doses between 10 and 100mg have been investigated in human volunteers and were well tolerated (personal communication).

3. Glycoprotein Ib Antagonists

Nonactivated platelets adhere at low shear rates to ligands in the subendothelial extracellular matrix which becomes exposed to the circulating blood during vascular injury: subendothelial von Willebrand factor attaches to GPIb/IIa of the platelet membrane.[56] At high shear rates, such as those found in arteries, platelets adhere to surface-bound von Willebrand factor via GPIb and then aggregate to Willebrand factor or to GPIIb/IIIa. Other subendothelial ligands such as fibronectin and vitronectin may also bind to other platelet receptors. Binding of GPIb receptors modifies GPIIb/IIIa receptors to their functional form. Fibrinogen and von Willebrand factor can then bind to exposed GPIIb/IIIa, resulting in platelet aggregation.

Monoclonal antibodies raised against GPIb, and synthetic GPIb receptor antagonists are effective in experimental thrombosis in different animal models.[57] Similarly, antibodies against multimeric von Willebrand factor also have antithrombotic properties.[58,59] A peptide corresponding to fragment amino acids 445-773 of von Willebrand factor blocks the GPIb receptor and thus prevents the interaction of von Willebrand factor with denuded subendothelium.[60] Also, inhibition of the multimerisation of von Willebrand factor with aurintricarboxylic acid has moderate antithrombotic properties.[61]

4. Thromboxane Synthase Inhibitors, Thromboxane Receptor Antagonists and Compounds with Dual Activity

4.1 Thromboxane Synthase Inhibitors

Thromboxane synthase inhibitors have been developed with the expectation of not only suppressing TXA_2 biosynthesis but also sparing or even enhancing the formation of prostacyclin by the vascular endothelium. Thromboxane synthase inhibition offers the advantage over aspirin-type cyclooxygenase inhibitors to reorient the arachidonic cascade towards an overproduction of inhibitory prostanoids (PGI_2, PGD_2) and a reduction of TXA_2 formation. However, specific inhibition of TXA_2 synthase produces an accumulation of cyclic prostaglandin endoperoxides which occupy and activate TXA_2 and endoperoxide receptors on platelets and endothelium and, thus, attenuate the inhibitory effect of PGI_2 and PGD_2.[62-65]

Most thromboxane synthase inhibitors have a moderate potency, a short duration of action, and do not result in a sufficiently sustained inhibition of TXA_2 production. Moreover, some individuals are poor responders to this type of drug. The increased generation of endoperoxides which share the same receptors as TXA_2 is a further problem which will not be solved by more potent and longer-acting drugs of this class (for review see Verstraete[66]).

Although thromboxane synthase inhibitors have shown some benefit in experimental models, their effects in clinical trials in patients with coronary artery disease have been disappointing.[66]

4.2 Thromboxane Receptor Antagonists

The more recently developed thromboxane receptor antagonists specifically impede the action of both TXA_2 and endoperoxides on their presumed common receptors on platelets and prevent vasoconstriction induced by TXA_2. These agents leave the normal pattern of thromboxane and prostacyclin formation unaltered. Thromboxane receptor antagonists prolong bleeding time more than thromboxane synthase inhibitors.[67] As expected, TXA_2 synthesis is not inhibited and PGI_2 generation is not augmented with specific thromboxane/endoperoxide receptor antagonists.

Some of the thromboxane/endoperoxide receptor blockers produce noncompetitive antagonism in human platelets because of their low dissociation rate. As a consequence, agonists equilibrate with the antagonist-occupied receptor pool so slowly that they fail to induce platelet activation. This property increases the potency and the duration of the receptor-blocking effect. Therefore, a low dissociation rate is an important measure of the effectiveness of receptor blockers in addition to their affinity.[68]

Clinical research with thromboxane receptor antagonists has been slow because the first compounds described had antagonistic effects on platelets but were agonistic on the vessel wall or vice versa.[65] Several of the thromboxane/endoperoxide receptor blockers are also relatively short-acting and the magnitude of their blockade is modest. This is particularly the case for daltroban but not so for vapiprost[69] and ifetroban (BMS 180291-A)[70] which are potent TXA_2 receptor blockers with a long duration of action.

Unfortunately, the initial clinical studies with vapiprost have been disappointing. The long plasma elimination $t_{1/2}$ of ifetroban is most interesting, as are its anti-ischaemic effects in canine models of pacing-induced ischaemia. However, no therapeutic studies with this compound have been published, and it remains to be demonstrated that such agents will offer a relevant advantage over aspirin.

4.3 Dual Action Agents

Ridogrel has a dual activity: it is a potent TXA_2 synthase inhibitor with modest additional TXA_2/PG endoperoxide receptor antagonist properties (at least 100-fold less) combined in one molecule.[71] The 2 properties were clearly demonstrated *in vitro*, in experimental animals, in human volunteers and in atherosclerotic patients. Ridogrel was shown to be effective in the prevention of arterial thrombosis induced by deep vessel wall injury in rats and in dogs.[72-74] Although the animal pharmacology is most promising, the preclinical evaluation is somewhat deceptive (for review see Verstraete[66]).

Picotamide, another molecular compound with dual properties, is a rather weak thromboxane synthase inhibitor and thromboxane receptor blocker.[75] There are even findings in humans suggesting that picotamide may inhibit platelet aggregation via mechanisms other than solely interference with thromboxane synthesis and activity.

5. Stable Analogues of Prostacyclin

The pharmacodynamic profile of iloprost mimics that of the endogenous prostanoid PGI_2. Both are potent inhibitors of platelet activation. Aggregation and release reactions stimulated by exposure to aggregating agents such as ADP, collagen or epinephrine (adrenaline) *in vitro* are essentially abolished by nanomolar concentrations of iloprost. Studies in animal models of vascular injury confirm a dose-related decrease in thrombogenesis similar to that of epoprostenol (synthetic PGI_2).[76] The putative mechanism for the antiaggregatory effect of iloprost involves platelet receptor-mediated activation of adenyl cyclase, which elevates intracellular levels of cAMP, thereby affecting phospholipase activity and cytosolic calcium. A decrease in receptor binding capacity but not in receptor affinity has been documented in human platelets exposed to iloprost *in vitro* and *in vivo*.[77]

With regard to other components of haemostasis, iloprost appears to have some fibrinolytic activity and can decrease neutrophil adhesion and

chemotaxis, but evidence of a substantial effect on blood fluidity is tenuous. There is significant inhibition of platelet aggregation (as measured *ex vivo*) during infusion of iloprost up to 2 ng/kg/min in healthy individuals and patients with peripheral vascular disease, but these effects decline rapidly once treatment is stopped.[78]

Iloprost is also an arterial vasodilator. However, the ratio of antiaggregatory to vasodilatory potency *in vivo* is in the order of 2 to 7 : 1, and this increases its therapeutic value for systemic administration. Of note, hypotension is a serious limitation of epoprostenol therapy. As in platelets, the mechanism of action in vascular preparations may involve an increase in smooth muscle cAMP secondary to receptor activation, but this is controversial.[79]

Iloprost inhibits constriction induced in various human and animal artery preparations by arachidonic acid, the thromboxane A_2 analogue U-46619, angiotensin II, phenylephrine and transmural neural stimulation. Intravital videomicroscopy of the everted hamster cheek pouch suggests that iloprost has salutary effects on microvascular tone and capillary perfusion.[79]

In humans, the drug decreases peripheral arterial vascular resistance and mean arterial blood pressure, with a mild increase in heart rate and cardiac index. It increases renal blood flow, but has a natriuretic effect which is independent of the haemodynamic change. Interestingly, it has been difficult to consistently demonstrate improved perfusion in the affected limb of patients with symptomatic peripheral ischaemia.[79] Ciprostene and taprostene are other stable prostacyclin analogues for intravenous use. More recently, several orally active stable PGI_2 derivatives (cicaprost, beraprost) have become available and are presently being evaluated in clinical trials.

6. Thrombomodulin

Thrombomodulin is an endothelial cell surface protein that forms a reversible 1 : 1 stoichiometric complex with thrombin. After formation of the complex, thrombin no longer has procoagulant activity but does acquire the potential to activate protein C 1000-fold compared with free thrombin.[81-82] Activated protein C, in the presence of protein S, inactivates blood coagulation factors Va and VIIIa.[83,84] Thus, by accelerating the activation of protein C, thrombomodulin plays an important role as regulator of coagulation at the surface of the vascular wall. Furthermore, thrombomodulin inhibits the proteolytic action of thrombin on macromolecular substrates, and thrombomodulin containing a galactosaminoglycan accelerates the inactivation of thrombin by antithrombin III.

Thrombomodulin is an integral membrane glycoprotein containing 575 amino acids and 5 domains. It is present on the vascular surface of endothelial cells of arteries, veins, capillaries and lymphatic vessels. The human thrombomodulin cDNA has been isolated[84] and expressed in Chinese hamster ovary cells.[85] This soluble human thrombomodulin contains only domains 1, 2 and 3 (amino acids 1-491) but not the transmembrane module and cytoplasmic tail of native single chain thrombomodulin.

Recombinant human soluble thrombomodulin is effective in the rat model of arteriovenous shunt thrombosis[86] and in disseminated intravascular coagulation models in mice and rat, also when the levels of antithrombin III are reduced. The presence of chondroitin sulfate on recombinant human soluble thrombomodulin results in a higher affinity for thrombin, a greater ability to inhibit thrombin-induced fibrin formation and platelet activation and is associated with antithrombin III-dependent inactivation of thrombin.[87,88] Administration of thrombomodulin prolongs the thrombin time, prothrombin time and activated partial thromboplastin time (APTT). Recombinant human thrombomodulin may be a means to generate endogenous activated protein C.

7. Protein C

Activated protein C is a natural coagulation inhibitor which plays a key role in the regulation of blood coagulation by selectively degrading coagulation cofactors Va and VIIIa, thereby inhibiting

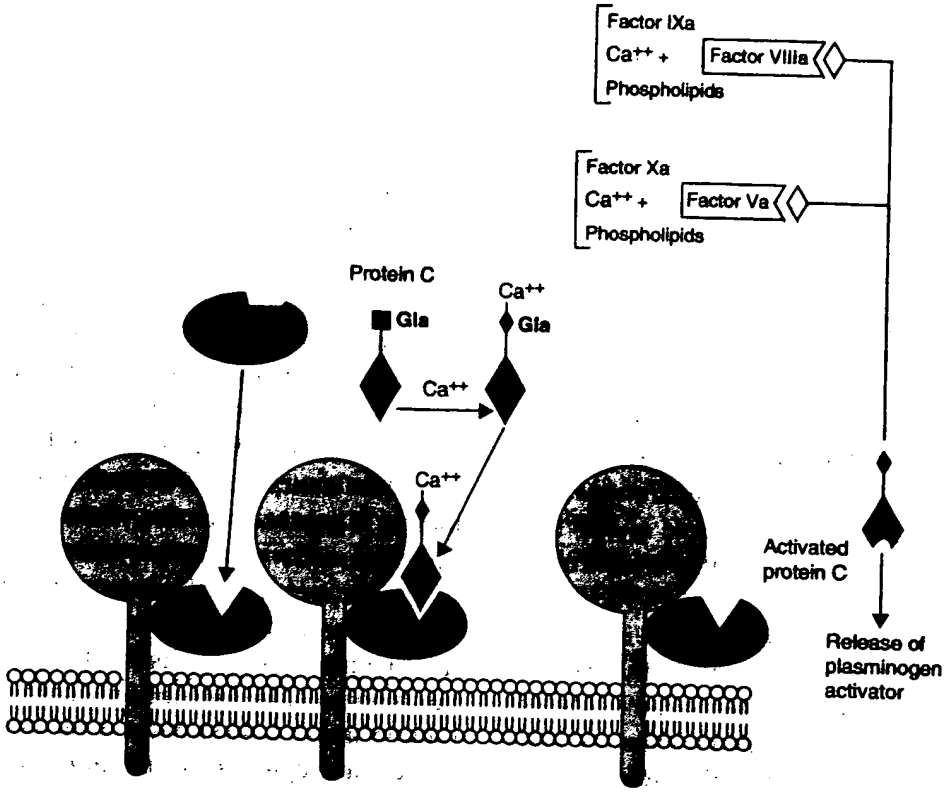

Fig. 1. Thrombin forms a complex with the endothelium-bound protein thrombomodulin (TM). This complex activates circulating protein C, which inhibits factor Va and VIIIa and releases tissue-plasminogen activator from the endothelial cells. Binding of activated protein C to phospholipids is facilitated by protein S. *Abbreviation:* Gla = γ-carboxyglutamic acid.

thrombus generation.[89] Thus, activated protein C stops the positive feedback actions of thrombin on the coagulation cascade (thrombin activates factors VIII and V), thereby limiting the coagulation process and thrombus propagation (fig. 1). Protein C is one of the vitamin K–dependent plasma proteins and is activated on the surface of intact endothelial cells by thrombin bound to thrombomodulin.

The anticoagulant effect of activated protein C is enhanced by protein S, which is another vitamin K–dependent plasma protein.[90] It has been reported that protein S increases the affinity of activated protein C for thrombogenic phospholipids approximately 10-fold, and that protein S abrogates the protective effect of factor Xa against activated protein C–mediated degradation of factor Va in the prothrombinase complex.

Human plasma contains 4 mg/L of protein C. The protein can be purified from plasma or obtained by recombinant technology.[91] Because of its endogenous origin and specificity of action, activated protein C is a potentially attractive antithrombotic agent. Earlier studies have shown that activated protein C inhibits disseminated intravascular coagulation in rabbits[92] and blocks the lethal effects of *Escherichia coli* infusion in baboons.[93] Moreover, human activated protein C has been shown to reduce jugular vein thrombus formation in dogs,[94] to delay the time to occlusion in anodal current–induced rat aorta thrombosis[95] and to reduce intermittent platelet thrombus formation in rat microvessels.[96] Human activated protein C inhibits thrombus formation on thrombogenic grafts interposed in arteriovenous shunts in baboons, when infused into the shunt just proximal to the thrombogenic site.[97]

Using a bovine protein C preparation in a microarterial thrombosis model in the rabbit, antithrom-

botic efficacy was demonstrated, but was associated with significant bleeding.[98]

8. Defibrotide

Defibrotide is the sodium salt of a single-strand polydeoxyribonucleotide of mean molecular weight 15 to 30kD, with a defined ratio of purine to pyrimidine bases of >0.85. It is prepared by controlled depolymerisation of DNA obtained from mammalian organs.

In most studies, particularly in those using a 3- to 4-fold higher dose than recommended in patients, defibrotide stimulates PGI_2 and PGE_2 production without changes in TXA_2 levels, and is associated with reduced leukotriene B_4 levels.[99] The drug also stimulates the fibrinolytic system, as shown by a decrease in the euglobulin lysis time and dilute clot lysis time. Furthermore, the lysis area of euglobulin on standard fibrin plates increases.[100,101] There are conflicting observations regarding the effect of defibrotide on activity in blood of tissue-type plasminogen activator (t-PA), plasminogen activator inhibitor type 1 (PAI-1) and α_2-antiplasmin.[102]

Defibrotide appears to be largely devoid of anticoagulant properties as determined by a lack of clinically significant effects on coagulation parameters, including APTT and the prothrombin time. The drug appears to have no effect on von Willebrand factor, factor VIII, factor Xa and prekallikrein, while its effects on antithrombin-III, fibrinogen and protein C require further confirmation.

Defibrotide has been reported to have little effect on platelet numbers, but may inhibit platelet function, possibly by stimulating the formation of PGI_2 or increasing platelet cAMP levels.

It has recently been shown that defibrotide stimulates expression of thrombomodulin in cultured human umbilical vein endothelial cells.[103]

Investigation of the pharmacokinetic behaviour of defibrotide is difficult because the drug is degraded in the body to a number of products, and the identity of the active derivative in humans is unclear. To date, the majority of pharmacokinetic data have been determined by following the fate of the carbohydrate moiety of the drug, 6-deoxyribose. Elimination of defibrotide in humans follows different kinetic models depending on the dose, with a 1-compartment model being the most appropriate following administration of low doses, and a 2-compartment model better suited following high doses. The elimination $t_{1/2}$ is short and increases with dose, with values of between 9.8 and 27.1 minutes after intravenous doses of 0.5 to 16 mg/kg or single intravenous injection of 200mg. The elimination $t_{1/2}$ appears to be independent of the route of administration, with similar values being obtained after oral and intravenous administration.

An antithrombotic action of defibrotide has been demonstrated in a number of animal models of venous thrombosis, in which the drug was able to attenuate the formation of collagen-, activated prothrombin complex-, thrombin- and mechanically induced venous thrombosis, and to reduce the size and alter the composition of those thrombi that are formed.[105]

Defibrotide has been demonstrated to be more effective than placebo for the prevention of postoperative deep vein thrombosis, but does not appear to be superior to subcutaneous unfractionated heparin.[106]

9. Tissue Factor Pathway Inhibitor

Tissue factor (TF) is an integral membrane protein of the vascular endothelium that functions as an essential cofactor for the proteolytic activity of factor VII towards its substrates, factors IX and X.[107,108] Human plasma contains a tissue factor pathway inhibitor (TFPI), formerly termed anticonvertin,[109] external pathway inhibitor (EPI)[110] or lipoprotein-associated coagulation inhibitor (LACI).[111]

This endogenous protease inhibitor is a 276-amino acid polypeptide consisting of 3 Kunitz-type serine protease inhibitor domains. The majority of human TFPI in plasma is associated with apolipoprotein AII, possibly via a mixed disulphide linkage, and interacts with the formation of fi-

brin in a 2-step fashion. In the first step, which is independent of calcium, TFPI binds to factor Xa by the second Kunitz domain, presumably through an active arginyl site. This reaction does not involve the Gla-residues on factor Xa. The bimolecule then induces a feedback inhibition of TF – factor VIIa – complex, thereby inhibiting the extrinsic pathway of coagulation. In this second step, the binding occurs through the first Kunitz domain. This reaction requires calcium, and the Gla-residues on factor Xa are essential.[111]

There is accumulating evidence that the TF-induced coagulation system is involved in arterial thrombosis and atherogenesis. Atherosclerotic plaques contain TF synthesising cells, and plaque rupture leads to exposure of TF activity to the circulating blood.[112] Elevated TFPI activity has been demonstrated in patients with MI,[113] even at young age,[114] suggesting that TFPI levels adapt to changes in the activity of factor VII.

Recombinant TFPI ($TFPI_{1-161}$) lacking the basic C terminal region and the third Kunitz domain has been produced in yeast cells.[115] This truncated form of TFPI in contrast to full length TFPI does not bind to heparin but has dose-related antithrombotic activity in experimental venous thrombosis induced in rabbits by a combination of destruction of endothelium and restricted blood flow.[116] Although $TFPI_{1-161}$ displayed a dose-dependent increase in activity in the anti–factor Xa, APTT and prothrombin time assays, APTT and prothrombin time assays were, for the same antithrombotic effect, much less prolonged compared with low molecular weight heparin. TFPI has no direct effect on thrombin and does not prolong the clotting time in the anti–factor IIa assay, even at high dose. No bleeding was observed in rabbits receiving $TFPI_{1-161}$ 10 mg/kg, an antithrombotic dose as effective as 60 anti–factor Xa IU/kg of tinzaparin sodium. Full length recombinant TFPI expressed in *E. coli*[117,118] completely prevented arterial reocclusion after vessel wall injury that yielded platelet-rich thrombi.[119]

Administration of recombinant TFPI may become an interesting antithrombotic drug targeted to exposed subendothelium.

10. Mixture of Low Molecular Weight Sulfate Glycosaminoglycans – Danaparoid Sodium

Danaparoid sodium is a low molecular weight heparinoid (6kD). It consists of a polydisperse mixture comprising sulfated glycosaminoglycans derived from animal mucosa, heparan sulfate (83% w/w), of which some 4 to 5% has high affinity for antithrombin III, dermatan sulphate (12% w/w) and a minor amount of chondroitin sulfate (5% w/w).[120]

There is uncertainty whether the low affinity fraction of danaparoid sodium has an antithrombotic function[121] or not.[122] Danaparoid sodium is more efficacious than heparin and is associated with less and shorter-lasting bleeding than heparin in various animal models of thrombosis.

The complex mechanism of the antithrombotic activity of danaparoid sodium can so far only be partially explained. Its anticoagulant profile is characterised by a high ratio of anti–factor Xa/antithrombin activity (14/<0.5) resulting in an effective inhibition of thrombin generation. The anti-Xa activity is mediated by antithrombin III and is not inactivated by endogenous factors that neutralise heparin. The low antithrombin activity is mediated by heparin cofactor II and antithrombin III. The heparin sulfate fraction with low affinity for antithrombin III, despite lacking significant effects on coagulation factors Xa and IIa (thrombin) *in vitro*, has been shown in animal studies to contribute substantially to the antithrombotic activity. In contrast to heparin, danaparoid sodium shows little or no effect on blood platelet function *in vitro* or *in vivo*.

Pharmacokinetic studies have been primarily based on the kinetics of relevant anticoagulant activities because no specific chemical assay methods are available. In comparison with heparin, danaparoid sodium has a prolonged elimination $t_{1/2}$ of anti–factor Xa activity. After intravenous and subcutaneous administration, the antithrombin activ-

ity $t_{1/2}$ of danaparoid sodium is shorter (1.8 ± 0.6h) than its anti–factor Xa $t_{1/2}$ (17.6 ± 1.1h). Danaparoid sodium has an absolute bioavailability of 100% after subcutaneous administration.

The kidneys play an important role in the elimination of the anti–factor Xa activity of danaparoid sodium, but cellular metabolism seems unlikely since the liver does not affect the anti–factor Xa activity, and there is only a slight and reversible binding to the endothelium.[123]

Danaparoid sodium has been shown to be effective in the prevention of deep vein thrombosis in patients with thrombotic stroke,[124] after elective hip surgery[125-127] or hip fracture.[128,129] Other clinical studies have been reviewed by Nurmohamed et al.[130] The long $t_{1/2}$ of danaparoid sodium, which is not effectively neutralised by protamine, has been clinically difficult to manage.

11. Sulodexide

Sulodexide is a substance extracted from pig intestinal mucosa. It is a highly purified preparation containing 2 principal components: a fast-moving iduronyl-glycosaminoglycan sulfate with a high affinity for antithrombin III (80%) and a dermatan fraction (dermatan sulfate) with an affinity for heparin cofactor II.[131]

In various animal models, sulodexide shows antithrombotic activity, probably by blocking platelet activation by thrombin, by an inhibitory effect on platelet adhesion[132] and through its anticoagulant and profibrinolytic activity.[133]

Antiatherosclerotic activity of sulodexide has been inferred[134,135] and may be related to a hypolipidaemic activity linked to lipoprotein-lipase release,[134] a more rapid catabolism of cholesterol-rich lipoproteins (very low density lipoproteins-low density lipoproteins (VLDL-LDL) by the liver[133] and an antiproliferative effect on smooth muscle cell types.

At therapeutic doses in patients, sulodexide inhibits activated factor X without influencing the APTT and thrombin time.[136,137] It also exerts profibrinolytic activity, evidenced by t-PA activation and PAI-1 inhibition.[138-141] Plasma and serum viscosity are also reduced by sulodexide.[141,142]

A multicentre study was conducted to assess the efficacy of sulodexide in preventing death and thromboembolic events after acute MI.[143] 3986 patients who had recovered from acute MI were randomised to receive either the standard therapy routinely in use in each study centre, with the exclusion of antiplatelet and anticoagulant drugs, or the standard therapy plus sulodexide. Between 7 and 10 days after the episode of acute MI, sulodexide was administered as a single daily intramuscular injection (600 lipoprotein lipase releasing unit; LRU) for the first month, followed by oral capsules (500 LRU twice daily). After 12 months, 7.1% of deaths were recorded in the control group and 4.8% in the sulodexide group (32% risk reduction, $p = 0.0022$). Furthermore, there was a 28% risk reduction in the sulodexide group ($p = 0.035$). A significant reduction in left ventricular thrombus formation was also observed in sulodexide recipients. Sulodexide was well tolerated and devoid of significant adverse events.

This study provides evidence that long term therapy with sulodexide started early after an episode of acute MI is associated with reductions in total mortality, rate of reinfarction and mural thrombus formation.

Several other glycosaminoglycans are being developed, but clinical experience is limited. The same holds for synthetic hypersulphated lactobionic acid amide (aprasulate).

12. Antithrombin III

The natural plasma protein antithrombin III is a relatively poor inhibitor of thrombin, but its inhibitory effect is increased 10 000-fold in the presence of heparin. *In vivo*, the interaction between antithrombin and heparin probably takes place at the endothelial cell surface where the protein binds to the heparin-like glycosaminoglycan, heparan sulfate. It has been shown that for tight binding of heparin to antithrombin III, a particular pentasaccharide sequence must be present.[144] This pentasaccharide brings about a change in confor-

mation in antithrombin, which involves an arginine residue[145] that is sufficient for near maximal acceleration of the inhibition of factor Xa.[146]

In contrast, much longer heparin species are required for significant acceleration of the rate of inhibition of thrombin by antithrombin III, since this reaction appears to be accelerated principally by simultaneous binding of both thrombin and antithrombin III to the same heparin molecule, increasing the inhibition rate of thrombin by antithrombin III encounter frequency.[146]

12.1 Human Plasma-Derived Antithrombin III

Concentrates of human plasma antithrombin III are available. As with all plasma products, the potential for viral infection must be weighed against the therapeutic benefits and alternatives. The concentrates contain some nonfunctioning, cross-reacting material, so the effect of therapy should be monitored by functional rather than antigenic antithrombin assays.[147] The biological $t_{1/2}$ of antithrombin from concentrates ranges from a mean of 61[148] to 92 hours.[149] The $t_{1/2}$ is not affected by the presence of coumarins[148] but is shortened by heparin[150] and in the postoperative period.[147]

Because of the moderate survival time of antithrombin III in the circulation, alternate day substitution is usually sufficient, although activity should be monitored regularly; it seems reasonable to maintain activity above 80% of the normal blood level during the treatment period.

In septicaemia, where there is widespread endothelial damage, the action of antithrombin III may be impaired. In addition, under conditions of a fulminant inflammatory response, as occurs during *E. coli* sepsis, the expression of heparin-like receptors on the vascular endothelium may be down-regulated in the same manner as the thrombomodulin and protein S receptors. This may in part explain why high concentrations of antithrombin III are necessary to prevent shock in animal models of sepsis.[151,152] Theoretically, combined antithrombin and heparin therapy should be more effective than antithrombin alone in the management of shock, but unfortunately this form of treatment did not improve the outcome in shocked patients and was associated with an increased risk of bleeding.

In humans, 2 randomised trials compared antithrombin III with a synthetic protease inhibitor[153] or antithrombin III with heparin.[154] Both studies documented a significant attenuation of disseminated intravascular coagulation (DIC) after antithrombin III treatment, but neither included a placebo control group. A placebo-controlled, double-blind trial in patients with septic shock and DIC, treatment with a plasma concentrate of antithrombin III achieved significantly earlier correction of DIC but failed to decrease mortality in a significant manner.[155]

Compared with heparin alone, adjunctive intracoronary therapy with a plasma antithrombin III concentrate does not appear to have any beneficial effect on procedural outcome as well as type and frequency of acute complications during PTCA, even in subgroups of patients with a high risk for thrombotic complications.[156] Thus, a local deficiency of antithrombin III does not seem to play a major role for the failure of heparin to abolish thrombotic complications during PTCA.

12.1 Recombinant Antithrombin III

Different recombinant antithrombin III molecules are now available. Numerous problems had to be solved because of the failure to obtain satisfactory expression of functional or reactivatable antithrombin III from *E. coli*.[157] Even expression in yeast gave only poor levels of active antithrombin.[158] The majority of reports on active wild-type and variant antithrombin III have thus involved mammalian cell or baculovirus expression. In addition to the greater time involved in obtaining protein from such a system compared with *E. coli*, there is the further problem of heterogenous glycosylation, which would be absent in bacterial expression. Differences in glycosylation of antithrombin have been shown to affect the affinity of the antithrombin for heparin.[159-161]

Studies on recombinant antithrombin III fall into 3 main categories: those on the wild-type in-

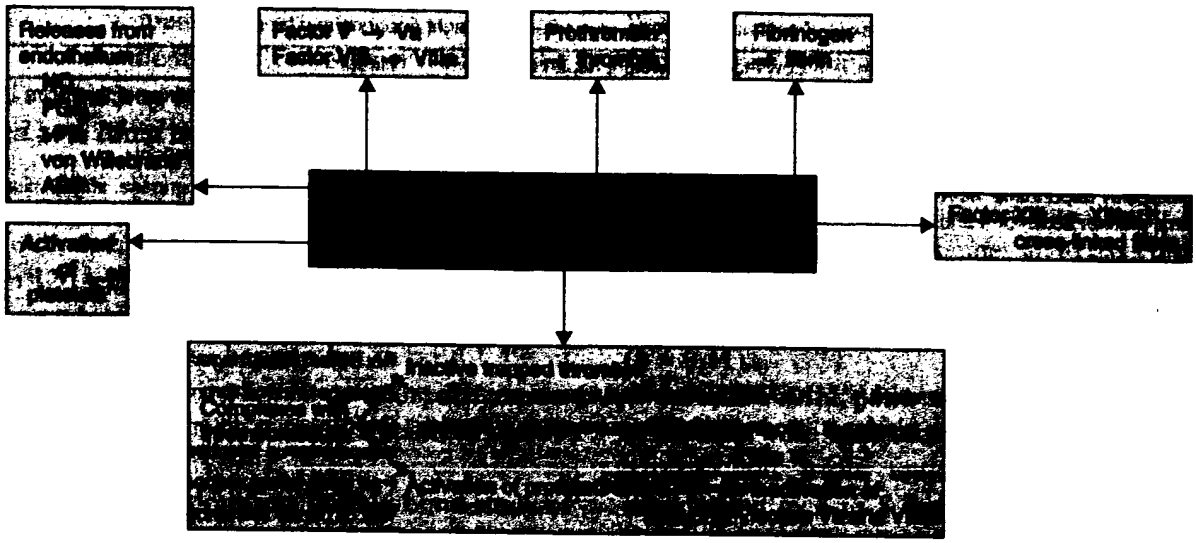

Fig. 2. Thrombin is the pivotal enzyme in coagulation, being responsible for positive feedback activation, rapid activation of platelets and endothelial cells, and indirectly via thrombomodulin, for its activation.

hibitor, those containing mutated residues in the reactive centre loop and thus involved in the formation of stable complex with proteinase, and those involving mutations in the heparin binding site.[162]

13. Direct Antithrombins

With the recognition of a central role of thrombin in the pathogenesis of acute coronary thrombosis (fig. 2), therapy with heparin, an indirect inhibitor of thrombin, has been used to the limit of its safety.[163,164] The direct antithrombins have emerged as a promising alternative to the intrinsic limitations of heparin.

The prototype of the direct antithrombins is hirudin, produced in the salivary glands of the European leech, *Hirudo medicinalis*. The anticoagulant properties of a secreted product of *H. medicinalis* were first recognised in 1884,[165] followed 20 years later by the isolation of a crude anticoagulant compound from the leech.[166] Although the first comparisons of the antithrombotic properties of crude hirudin preparations with those of heparin were published in 1927,[167] it was only in the late 1950s that Markwardt purified the leech anticoagulant sufficiently to recognise and characterise its polypeptide structure.[168]

Over 100 years after its discovery, large-scale production of recombinant hirudin has led within the past 7 years to the development, preclinical evaluation and introduction into clinical trials of hirudin and other direct antithrombins.

13.1 Hirudin

Natural hirudin is a single-chain, carbohydrate-free polypeptide containing 3 intramolecular disulphide bridges and a sulfated tyrosine residue. The polypeptide chain contains 65 amino acids with a molecular weight of approximately 7kD. Recombinant hirudin has been obtained using *E. coli* and yeast. With both methods, hirudin is expressed as desulfatohirudin lacking the sulfate residue on tyrosine 63 (desirudin). The importance of the latter is controversial, since the anticoagulant activity of natural and recombinant hirudin appears to be similar.[169]

Unlike heparin, which requires endogenous cofactors for activity (mainly antithrombin III and heparin cofactor II), hirudin does not need a cofactor for its anticoagulant activity and is therefore still active when there is a deficiency of these pro-

Table II. Comparison of some properties of unfractionated heparin, low molecular weight heparins and hirudin

Unfractionated heparin	Low molecular weight heparins	Hirudin
Inhibits thrombin and factor VII to the same extent, much less factors IXa and XIa	Inhibits mainly factor Xa, and to some extent thrombin	Specific and potent inhibitor of thrombin
Antithrombin III-dependent	Antithrombin III-dependent	Antithrombin III-independent
Neutralised by heparinase, several plasma proteins, platelet activator 4 and endothelium	Neutralised by heparinase, weak endothelium binding	Not neutralised by heparinase, endothelium, macrophages, fibrin monomer, plasma proteins and platelet activator 4
Does not inactivate clot-bound thrombin and factor VII	Does not inactivate clot-bound thrombin and factor VII	Inactivates clot-bound thrombin
Inhibits platelet function	Inhibits platelet function	Prevents thrombin induced aggregation but not other platelet agonists
Induced thrombocytopenia is not rare	Can induce thrombocytopenia	Does not induce thrombocytopenia
Bioavailability after subcutaneous injection is 30%	Bioavailability after subcutaneous injection is >90%	Good bioavailability (≈80%) after subcutaneous injection
Poor dose-effect response	Fair dose-effect response	Fair dose-effect response
Not immunogenic	Not immunogenic	Not or barely immunogenic
Transient increase of liver enzymes is common	Transient increase of liver enzymes is possible	No liver toxicity
Increases vascular permeability	No increase in vascular permeability	No increase in vascular permeability

teins or their activity inhibited. Hirudin is a specific inhibitor of thrombin to which it binds near the active centre at the substrate recognition site with extraordinary tightness. In addition, there are extensive close contacts between hirudin and thrombin over an extended area of the molecule, forming a highly stable noncovalent complex. All known functions of thrombin are inhibited. Hirudin is compared with heparins in table II.

The terminal $t_{1/2}$ of recombinant hirudin in healthy young volunteers is 50 to 65 min[170,171] with a $t_{1/2}$ of its effect on the APTT of about 2 hours.[89,172-174] In contrast, in older patients with established coronary artery disease with normal renal function (serum creatinine 1.0 ± 0.2 mg/dl), the plasma $t_{1/2}$ of hirudin was found to be 2 to 3 hours,[175] in agreement with the $t_{1/2}$ of the effect of hirudin on the APTT of about 2 to 3 hours.[175] In these patients, plateau/baseline APTT ratios were 1.5, 2.0, 2.3, 2.7 and 2.9 (all ± 0.1), respectively, with hirudin infused at 0.02, 0.05, 0.1, 0.2, and 0.3 mg/kg/h without prior bolus. 62 to 77% of the APTT plateau value was seen within 30 minutes after starting the infusions and was directly related to the dose. Plasma hirudin concentrations correlated well with the APTT/baseline ratios (r = 0.88), but less so with the activated coagulation time (ACT)/baseline ratios (r = 0.44). Plasma concentrations of hirudin and ACT in seconds correlated well overall (r = 0.80), but considerable overlap occurred between baseline ACT and ACT at plasma hirudin concentrations of <1 mg/L. Prothrombin times were significantly prolonged only at a dosage of 0.05 mg/kg/h or greater and were 11.8 ± 0.5 [international normalised ratio (INR) = 1.0] and 15.8 ± 0.9 (INR = 2.3) seconds for the 0.05mg and 0.3mg doses. Thrombin times were beyond >600 seconds at 6 hours in virtually all patients.

Bleeding times were not significantly prolonged in this trial and only mildly prolonged during administration of r-hirudin in another study.[176] Recombinant hirudin appears to be a weak allergen, and hirudin-specific IgE antibodies were rarely seen in 163 immunocompetent healthy volunteers receiving recombinant hirudin twice at a 1-month interval.[177] No adverse effects occurred. No antibodies to hirudin were detected 2 weeks after the infusion. The APTT appeared most suited for monitoring recombinant hirudin administration.

In a double-blind pilot trial 113 low-risk patients with stable angina pectoris undergoing PTCA were randomised to a 24-hour infusion of

either recombinant hirudin (desirudin) or heparin.[178] All patients received aspirin for at least 4 weeks beginning on the day of PTCA. Hirudin was given as a 20mg bolus followed by an infusion of 0.16 mg/kg/h and compared with heparin given as a 10 000IU bolus followed by 12 IU/kg/h. The dosage of both drugs was adjusted to a target APTT of 85 to 120 sec. Acute closure, leading to MI and/or coronary bypass surgery, occurred in 10.3% of patients randomised to heparin but in only 1.4% of patients receiving hirudin. None of the differences in this small pilot trial reached statistical significance.

A European randomised, double-blind study of hirudin (40mg bolus followed by 0.2 mg/kg/h) and heparin is terminated and the results are being analysed (HELVETICA). The primary end-point is event-free survival (freedom from cardiac death, MI, coronary bypass surgery, bail-out procedure, repeat PTCA or elective stent placement) within 30 months of PTCA.

In a multicentre, open-label pilot trial, 166 patients with unstable angina and angiographic thrombus were randomised to a 72- to 120-hour infusion of heparin (n = 50) or recombinant hirudin (n = 116) at 5 escalating dosages (0.05 mg/kg/h to 0.3 mg/kg/h).[179] A 0.9 mg/kg bolus preceding the 0.3 mg/kg/h dose was abandoned because of minor bleeding, and was replaced by a 0.6 mg/kg bolus. Heparin was given as a 5000IU bolus, followed by an infusion of 1000IU/h, which was adjusted to an APTT of 65 to 90 sec or 90 to 110 sec. Hirudin was not adjusted to the APTT. All patients received aspirin and triple anti-ischaemic therapy. As in other studies, patients assigned to hirudin were in the APTT target range more often than those assigned to heparin (71 versus 16%, $p < 0.0001$). APTT prolongations with hirudin at 0.2 and 0.3 mg/kg/h were not significantly different.

Upon repeat angiography at 72 to 120 hours, patients assigned to hirudin (compared with heparin) had an improved cross-sectional area of the culprit vessel ($p = 0.08$), a larger minimum cross-sectional area ($p = 0.028$) and a lesser diameter stenosis ($p = 0.071$). The improvement in TIMI flow grade was not significant ($p = 0.44$). Importantly, equal angiographic benefit was seen with hirudin at 0.1 to 0.3 mg/kg/h, suggesting a plateau effect for the benefit of hirudin in this dose range. At similar APTT prolongations, hirudin improved the angiographic endpoints compared with high dose heparin. Clinical outcomes at 30 days did not reach statistical significance, although MI developed in 2% of hirudin and 8% of heparin recipients ($p = 0.11$).

In GUSTO-IIa, patients with an acute coronary syndrome, who also received thrombolytics and aspirin and triple antianginal therapy, were randomised to 3 to 5 days of intravenous fixed-dose hirudin (0.2 mg/kg/h) or weight-adjusted heparin. Although efficacy data are not expected until late 1995, the incidence of intracerebral bleeding was considerably increased in both hirudin and heparin recipients compared with previous trials in patients receiving heparin. The overall incidence of intracerebral bleeding in GUSTO-IIa was 1.3% in 620 heparin recipients and 0.7% in 644 hirudin recipients, while in GUSTO-1 the incidence in 30 892 heparin recipients was 0.6%.[164]

In the open-label TIMI-5 pilot study, patients with acute MI were treated with front-loaded alteplase plus aspirin and randomised to either heparin or hirudin. 162 patients received a 5-day infusion of escalating hirudin dosage (0.05 to 0.2 mg/kg/h), and 84 patients received heparin adjusted to an APPT of 65 to 90 seconds.[180] Although the difference in TIMI grade 2 and 3 flow at 90 minutes was not significantly different between hirudin and heparin (82.1 versus 78.6%, respectively), it reached significance at 18 to 26 hours (97.8 versus 89.2%, $p < 0.01$) because of a decrease in reocclusion rates (1.6 versus 6.7%, $p < 0.07$) in patients receiving hirudin, and a higher rate of reperfusion in the hirudin group. Major spontaneous haemorrhage occurred in 4.7% of heparin versus 1.2% of hirudin recipients. Intracranial haemorrhage occurred in 1 heparin patient. Patients in the TIMI-6 pilot trial were treated with streptokinase and aspirin and were randomised to hirudin or heparin.[181] A trend towards im-

proved outcome was observed with the higher dosage of hirudin (0.1 and 0.2 mg/kg/h) compared with the lower dosage (0.05 mg/kg/h) of hirudin and heparin.

The high bleeding risk, particularly of haemorrhagic stroke, of high-dose hirudin and heparin became apparent in 3 large phase III trials. In TIMI-9A[163] and GUSTO-IIa,[164] hirudin was administered as a 0.6 mg/kg bolus followed by a fixed-dose infusion of 0.2 mg/kg/h for 96 hours (TIMI-9A) or 72 to 120 hours (GUSTO-IIa). In HIT-III,[182] patients were randomised to a 48 to 72h infusion of either hirudin (0.4 mg/kg followed by 0.15 mg/kg/h) or heparin. A front-loaded t-PA (alteplase) protocol was used in all trials. In HIT-III, but not in GUSTO-IIa and TIMI-9A, adjustment of the study drug to an APTT prolongation of 2 to 3.5 times baseline was recommended. In addition to front-loaded alteplase (or streptokinase in 15% of TIMI-9A patients), all patients received aspirin.

In contrast to GUSTO-I, where 50% of patients had APTTs below the target range of 60 to 85 seconds, a weight-adjusted heparin regimen was used in GUSTO-IIA and TIMI-9A (patients <80kg and ≥80kg received heparin 1000 and 1300 IU/h, respectively) and heparin was titrated to an APTT target range of 60 to 90 seconds. Compared with GUSTO-I, this strategy resulted in a 20% increase in the total amount of heparin given.[164] Heparin in HIT-III was weight-adjusted on a per-kilogram basis: 70 IU/kg followed by 15 IU/kg/h.[182]

Intracranial bleeding in TIMI-9A occurred in 1.7% of hirudin and 1.9% of heparin patients. Major spontaneous noncerebral haemorrhage occurred in 7.0 and 3.0%, respectively ($p < 0.02$). A baseline creatinine of >1.5 mg/dl, older age, lower bodyweight and higher APTT levels (100 versus 86 seconds in nonstroke patients) were associated with bleeding in hirudin patients, suggesting that reduced renal clearance of hirudin contributed to the higher incidence of bleeding.

In GUSTO-IIa the dosages of hirudin and heparin were identical to those of TIMI-9A. The overall haemorrhagic stroke rate in thrombolysis patients was 1.8%; it was 2.2% with hirudin and 1.5% with intravenous heparin ($p = 0.34$). There was a trend towards increased intracerebral bleeds with hirudin when given with streptokinase (3.2%) compared with alteplase (1.7%) and an increased risk with age, female gender and greater APTT prolongation at 12 hours of 110 and 87 sec, respectively, in patients with and without stroke, $p = 0.031$). This contrasts with the haemorrhagic stroke rates in GUSTO-I, which were 0.57% with intravenous heparin plus streptokinase and 0.7% with intravenous heparin plus alteplase. The incidence of haemorrhagic stroke in GUSTO-IIa patients not receiving thrombolysis was also high: 0.3% of all patients with cerebral bleeding were in the hirudin group. Haemorrhagic strokes in the thrombolytic group occurred at a median time of 8 hours after start with hirudin and after 17 hours with heparin (no significant difference).

The HIT-III trial was stopped after an imbalance in the incidence of haemorrhagic stroke became apparent, which was 3.4% in hirudin recipients ($n = 148$) versus none in heparin patients ($n = 154$). The incidence of confirmed cardiac rupture was 2% in hirudin versus 0.6% in heparin patients. All haemorrhagic strokes (all on hirudin) occurred within the first 24 hours after treatment start (3 within the first 6 hours, 1 at 13 hours and 1 at 23 hours). Patients on hirudin who bled had a median APTT of 106 seconds, versus 76 seconds in those who did not bleed.

The early plasma hirudin concentrations produced by the 0.6 mg/kg bolus were in excess of what has been predicted in a phase I trial of patients with stable coronary artery disease and serum creatinine of 1.0 ± 0.2 mg/dl, where 62 to 77% of the plateau APTT prolongation was achieved within 30 minutes after start of a maintenance infusion of hirudin without bolus administration.[175]

In view of these results, and the observation that hirudin at 0.1 mg/kg/h appeared to be as efficacious as higher doses of hirudin, both in the unstable angina[183] and the TIMI-5[180] pilot studies, GUSTO-IIb and TIMI-9B have now restarted at a lower hirudin dosage (0.1 mg/kg bolus followed by 0.1

mg/kg/h) and heparin dosage (1000 IU/h) without weight adjustment). In addition, both the heparin and the hirudin infusion are now adjusted to a target APTT range of 55 to 85 seconds (TIMI-9A) and 60 to 85 seconds (GUSTO-IIa) to avoid APTTs >100 seconds, which are clearly associated with increased risk of intracerebral haemorrhage. Down-titration of hirudin and adjustment to an APTT prolongation of 2 to 3 times baseline may take better advantage of the lower anticoagulant to antithrombotic ratio of hirudin compared with heparin, which was clearly established in preclinical studies, where hirudin was more effective than heparin at APTT ratios several-fold lower than those achieved with high dose heparin.

13.2 Hirulog

Hirulog is a bifunctional 20-amino acid peptide designed on the structure of hirudin. It combines a fragment of the C-terminus of hirudin (interacting with the anion-binding exosite of thrombin) with an N-terminus fragment [D-Phe-Pro-Arg-Pro-(Gly)], which interacts with the catalytic site of thrombin. The K_D of hirulog towards thrombin is 2.3×10^{-9} mol/L.[184,185] The hirulog-thrombin complex is only transient, as thrombin can slowly cleave the Pro-Arg bond in the N-terminal extension. This metabolic cleavage contributes to its $t_{1/2}$ on the APTT of about 40 minutes.[187,188] Only 20% of hirulog is excreted in the urine. Newer hirulogs have been synthesised with noncleavable bonds for hirulog. There is no antidote.

Phase I studies revealed a dose-dependent prolongation of the APTT with a 15 minute intravenous infusion of hirulog 0.05 to 0.6 mg/kg, resulting in APTTs from 1.7 ± 0.08 to 2.8 ± 0.55 times baseline. There was a good, though not linear, correlation between APTT and hirulog plasma concentrations. Thrombin and prothrombin times were not useful in titrating the dose of hirulog. Bleeding times were not significantly prolonged. When 0.3 mg/kg/h of hirulog was infused over 12 or 24 hours, peak APTT ratios were 2.1 to 2.5.[186]

In another dose-finding study in patients undergoing cardiac catheterisation, a good correlation was found between the APTT and plasma hirulog concentrations (r = 0.77).[187] The APTT was prolonged to 1.8 and 2.2 times baseline, respectively, 15 minutes after starting hirulog at 0.05 mg/kg followed by 0.2 mg/kg/h and 0.15 mg/kg followed by 0.6 mg/kg/h. No major haematoma or thrombotic complications occurred at the dosages tested. Fibrinogen peptide A (FPA) levels were suppressed during hirulog administration at dosages which, compared with heparin, caused less elevation in APTT, prothrombin time and ACT.

In a dose-escalation pilot study, Lidon et al.[188] evaluated hirulog in 55 patients with unstable angina, who also received aspirin and triple antiischaemic therapy. Hirulog was administered in escalating dosages of 0.02 to 0.5 mg/kg/h, increased every 30 minutes, for 72 hours. With dosages up to 1 mg/kg/h, only 1 of 20 patients experienced recurrent chest pain. The APTT in angina-free patients averaged 55.6 ± 6 seconds. Plasma FPA levels were suppressed at dosages of 0.25 to 0.5 mg/kg/h. The APTTs fell to baseline 4 hours after discontinuation of hirulog. There was no rebound elevation of FPA at that time. Occult faecal blood was noted in 3 patients.

In a second dose-finding study, hirulog 0.25 to 1.0 mg/kg/h given for 72 hours significantly reduced the rate of death or nonfatal reinfarction compared with a dosage of 0.02 mg/kg/h.[189]

Pilot trials have evaluated hirulog as an adjunct to thrombolysis. Lidon et al.[190] randomised 45 patients to hirulog (0.5 mg/kg/h without prior bolus, reduced to 0.1 mg/kg/h after 12 hours) or heparin (1000 IU/h) added to streptokinase. At 90 and 120 minutes TIMI 2 and 3 flow was observed in 77 and 87% of patients treated with hirulog and heparin. TIMI grade 3 flow was present at 120 minutes in 77% of hirulog versus 40% of heparin patients.[190] In patients receiving heparin plus streptokinase, the corresponding rates of TIMI 2 and 3 flow were 47% for both time points ($p < 0.5$ for the 90- and $p < 0.01$ for the 120-minute point). No reocclusion or recurrent angina were observed in the hirulog group 3 to 7 (mean 4.7) days later. Bleeding complications occurred in 12% of hirulog versus 27%

of heparin recipients (no significant difference). There was only 1 intracerebral haemorrhage, which occurred in the heparin group. APTTs peaked at 3 and 4 times baseline, respectively, with hirulog and heparin, probably secondary to the fibrinolytic effect of streptokinase, as plasma concentrations were not higher than predicted from phase I studies.

Angiographic patency of the culprit coronary artery lesion was assessed 90 and 120 minutes after the initiation of streptokinase and aspirin and again after 4±2 days in 68 patients with acute MI.[191] Patients were randomised to hirulog 0.5 mg/kg/h for 12 hours followed by 0.1 mg/kg/h (low dose), hirulog 1 mg/kg/h for 12 hours then placebo (high dose), or to heparin 5000U bolus then 1000 U/h titrated to an APTT 2 to 2.5 times control after 12 hours. At 90 minutes, TIMI grade 2 or 3 was observed in 96% of low dose hirulog recipients versus 79% of high dose hirulog and 46% of heparin recipients ($p = 0.006$). Respective TIMI 3 flow grade rates were 85, 61 and 31% of patients ($p = 0.008$). At 120 minutes, respective TIMI 2 or 3 rates were 100, 82 and 62% ($p = 0.046$), and TIMI 3 rates were 92, 68 and 46% ($p = 0.014$). At 90 minutes the relative risk for restoring TIMI flow grade 3 was 2.77 with low dose hirulog compared with heparin ($p < 0.001$) and 1.4 compared with high dose hirulog ($p = 0.04$).

Patients who received a placebo infusion after 12 hours experienced more clinical events and reocclusion during the following 4 days than patients in the other groups. In this trial, hirulog yielded higher early patency rates than heparin when given as adjunctive therapy to streptokinase and aspirin in the early phase of acute MI. High hirulog doses are unnecessary and may be less effective than lower doses. This suggests that too much thrombin inhibition may be harmful.

In a multicentre trial, Topol et al.[179] evaluated hirulog in 291 patients undergoing elective angioplasty and pretreated with aspirin. Following bolus administration, a 4-hour infusion of hirulog 0.6 to 2.2 mg/kg/h was given. Although there was a trend towards a dose-related increase in APTT prolongation, there was a wide overlap between APTT at different dosages. No statistically significant ACT level was associated with complete prevention of acute closure. There was no prolongation of bleeding time, and no patient developed life-threatening bleeding. Acute closure within 24 hours was inversely related to the hirulog dosage, and was 3.9% for the 1.8 and 2.2 mg/kg/h dosages combined.[179]

13.3 Hirugen

Modelled on the C-terminal fragment of hirudin, hirugen is a dodecapeptide comprising the 12 terminal residues of hirudin in an ion-binding blockade. It contains sulfated tyrosine to increase its thrombin activity. Hirugen inhibits thrombin, forming an inhibitor complex of substantially lower affinity compared with hirudin (K_D 1.5×10^{-7} mol/L versus 0.2×10^{-12} mol/L for hirudin). *In vitro*, hirugen competitively inhibited thrombin-mediated fibrinogen cleavage and platelet activation.[192,193] In experiments with exteriorised arteriovenous shunts in baboons, hirugen prevented *ex vivo* platelet deposition in low-shear flow chambers connected to chronic arteriovenous shunts of baboons, but failed to affect *ex vivo* platelet deposition on collagen type I–coated tubing at a dose of 75 mg/kg (APTT 4-fold baseline).[192]

Since hirugen inhibits platelet activity *in vitro*, and contains the thrombin-recognising peptide sequence present in the platelet thrombin receptor,[194] higher doses of hirugen could in theory inhibit platelet deposition on highly thrombogenic surfaces as well. At present, no clinical studies with hirugen are under way as its antithrombotic activity is much less potent compared with hirudin or hirulog.

13.4 Argatroban

Argatroban, an arginine-derivative which binds to thrombin with intermediate affinity (K_D 3.9×10^{-8} mol/L) competitively inhibits fibrinogen cleavage[195] and platelet activation by thrombin. Compared with heparin, argatroban is significantly more effective in the prevention of platelet-rich

thrombi after vascular injury, and was effective at APTTs of only 2 to 3 times baseline control.[196,197]

In a whole-blood thrombolysis study with stenosed femoral arteries in the rabbit, argatroban (100 μg/kg/min, APTT 2.5- to 3.0-fold baseline) accelerated reperfusion compared with heparin (200 IU/kg, APTT >5-fold baseline), although the incidence of reflow was not increased compared with heparin. Addition of aspirin did not accelerate thrombolysis by either argatroban or heparin.[199]

In a whole-blood clot thrombus model in stenotic canine coronaries, pretreatment with argatroban 200 μg/kg/min (APTTs 6 to 7.6 times control) significantly reduced the time to lysis by alteplase to 23 minutes compared with 40 minutes in the aspirin group. Addition of aspirin to argatroban did not shorten time to lysis, but reduced the incidence of reocclusion by platelet-rich thrombi from 75 to 20% relative to argatroban alone.[37] Argatroban was as effective in this model as abciximab in inhibiting the platelet GPIIb/IIIa receptor.

In a platelet-rich coronary thrombus model after endothelial injury created by electric current, acceleration of lysis by alteplase was observed in dogs pretreated with argatroban at a lower dosage (41 μg/kg/min). However, abolition of cyclic flow reductions caused by intermittent platelet aggregates required the addition of a TXA_2/PG endoperoxide receptor antagonist.[198]

The result with argatroban plus aspirin seems to suggest that additional platelet (thromboxane) inhibition may be necessary, when antithrombins of lower affinity are used to prevent early reocclusion after lysis with alteplase. On the other hand, high-affinity antithrombins like hirudin may be as effective as (or more effective than) antithrombins plus thromboxane blockade, without additional antiplatelet agents.[199,200]

Argatroban combined with aspirin was well tolerated in humans at a dose yielding a mean APTT 1.6 times baseline and did not prolong the bleeding time. Whether argatroban is effective at this dosage remains to be seen.

13.5 DUP-714

This boroarginine tripeptide binds to thrombin with moderately high affinity (K_D 4.1 × 10^{-11} mol/L) and inhibits thrombin-mediated platelet activation and fibrinogen cleavage.[201] DUP 714 reduced the incidence of venous thrombi in rabbits from 100% (controls) to 33%, and arteriovenous shunt thrombosis from 72 to 11%. Its anticipated potential for oral administration has not been borne out in experimental or human studies. Because of liver toxicity, presumably related to the boro constituent, human studies were not pursued further.

13.6 Other Direct Antithrombins

A variety of other antithrombins have been synthesised but no human trials have been planned. Among them, RWJ-27755 has been most extensively evaluated and has potent antithrombotic properties, as reviewed elsewhere.[200] Its alkylating properties have considerably tempered the enthusiasm for its clinical development.

Other antithrombins still in development are the thrombin receptor antagonist peptides[202] and hirudisins – hirudin derivatives combining IIb/IIIa receptor and thrombin inhibition. In the hirudisins, residues 32 to 35 of hirudin have been replaced by the integrin motif RGDS and KGDS, creating a potent thrombin inhibitor (K_D 0.16 to 0.26 × 10^{-12} mol/L compared with 0.2 × 10^{-12} mol/L for hirudin) with additional disintegrin activity.[203] In addition to inhibiting GP IIb/IIIa receptor-dependent platelet interactions, the platelet-binding integrin motif is expected to target the antithrombin action of hirudin to platelets, possibly allowing lower and safer doses of hirudin in the treatment of thrombotic disease.[205]

Similarly, hirudin targeted to fibrin by coupling it to Fab portions of antibodies directed against platelet GPIIb/IIIa receptor or fibrin β-chain[204] may allow for highly efficient antithrombosis at dosages lower than those currently required for hirudin.

14. Factor Xa Inhibitors

Direct thrombin inhibitors do not affect thrombin generation, and may leave some 'escaping' thrombin molecules unaffected. Inhibition of factor Xa can prevent thrombin generation and disrupt the thrombin feedback loop that autoamplifies thrombin production. Thus, inhibition of coagulation enzyme earlier in the cascade may be a rewarding approach.

14.1 Recombinant Tick Anticoagulant Peptide

Recombinant tick anticoagulant peptide (TAP), originally isolated from the soft tick *Ornithodoros moubata*, is a slow-binding, specific stoichiometric factor X inhibitor of 6.850kD. The 60-amino acid peptide has limited homology to the primary sequence of the Kunitz-type inhibitor family and binds tightly, but reversibly, to factor Xa with a K_D of 0.18 to 0.3 nmol/L.[205] Binding of TAP to factor Xa involves initial binding to a low-affinity 'exosite' (distinct from the catalytic site of factor Xa) followed by high-affinity interaction with the factor Xa catalytic site.[206] The inhibition constant of recombinant TAP towards factor Xa is 0.2 nmol/L.

TAP has been superior to heparin in the prevention of venous thrombi initiated by a local injection of a thromboplastin/blood mixture in rabbits[206] and in preventing heparin-resistant platelet thrombi in a primate model of arteriovenous shunts grafted with collagen and endarterectomised carotids.[207] Similar to previous studies with recombinant activated protein C,[98] APTT and template bleeding times were only minimally elevated with TAP.

TAP has been studied as conjunctive treatment with alteplase. In a comparison in a canine coronary model of platelet-rich thrombosis and stenosis, the time to reperfusion by alteplase was shortened from 40 minutes in animals receiving heparin 200IU/kg compared with 23 minutes with TAP 6 mg/kg/h and 20 minutes with hirudin 6 mg/kg/h. The incidence of reocclusion in this model was lower in the TAP group than in the hirudin group (3 of 8 versus 6 of 8 animals). Peak APTTs were 2.1 times baseline in the TAP and 13 times baseline in the hirudin group, while template bleeding times were 2.7 and 5.1 times control, respectively, in the TAP and hirudin groups.[208]

In interpreting the slight elevation of the APTT with antithrombotic doses of TAP, the exquisite sensitivity of the APTT assay to inhibitors of thrombin and the relatively slow kinetics of factor Xa inhibition by TAP need to be kept in mind. Thus, APTT may not be optimal for monitoring anticoagulation with TAP. Irrespective of this, reocclusion in the hirudin group occurred only after termination of the hirudin infusion in this study. However, in a second study in this model, TAP prevented reocclusion more efficiently than hirudin at equimolar plasma concentrations.[210]

These studies suggest that halting ongoing generation of thrombin has beneficial effects on vessel patency after thrombolysis. The observation that procoagulant factor Xa (like thrombin) is adsorbed onto fibrin strands of clots underscores the complexity of prothrombin activation *in vivo* and provides a further rationale for the efficacy of factor Xa inhibitors as antithrombotic agents.[210] High concentrations of TAP (5 μmol/L) were required to prevent FPA generation by clot-bound Xa *in vitro*. Heparin pentasaccharide did not inhibit clot-associated factor Xa activity at concentrations as high as 5 mg/L.[210]

Overall, these studies indicate that TAP, which has low immunogenicity, has considerable potential as antithrombotic treatment for the prevention of heparin-resistant arterial thrombosis and as conjunct with thrombolysis. With the emergence of a narrow therapeutic window of the direct thrombin inhibitors, the clinical development of TAP, despite its high costs, may become an option for the future.

14.2 Antistasin

Antistasin is a 15kD peptide isolated from the salivary glands of the Mexican leech *H. officinalis*.[211] It is a tight-binding and specific inhibitor of factor Xa. Antistasin (13kD) has potent anticoagulant activity for more than 30 hours after a single subcutaneous injection. This compound has

been studied in a variety of thrombosis models and found superior to heparin in preventing platelet-rich thrombi in dacron-grafted arteriovenous femoral grafts[212] and as effective as heparin in suppressing FPA levels in a rhesus monkey DIC model.[213] Template bleeding times were not elevated. Antistasin was also superior to heparin when used as adjunct to alteplase in a canine model of femoral arterial thrombosis.[214] Because of its strong immunogenicity, clinical development of antistasin is unlikely for the moment.

14.3 Synthetic Pentasaccharide

The pentasaccharide Org 31540/SR 90107A is a new sulfated pentasaccharide obtained by total chemical synthesis.[215] It is, however, chemically identical with the antithrombin III binding site found in heparin and some low molecular weight glycosaminoglycans. The complex displays antithrombotic activity by virtue of its potentiation of the antifactor-Xa activity of antithrombin III.[215,216]

This compound acts on free factor Xa and not on thrombin decay, and has been shown to possess antithrombotic efficacy in *in vitro* thrombosis models[217] and in animal experiments.[218,219] In the arteriovenous model, the pentasaccharide reduces thrombus growth by preventing platelet deposition in rats[220] and primates.[221] If antithrombotic doses are expressed in anti-Xa units/kg, the pentasaccharide is less potent than unfractionated heparin; however, if doses are expressed in mg/kg, the pentasaccharide shows more or less the same potency as unfractionated heparin. The duration of action, as measured by anti-Xa levels, is longer than that of unfractionated heparin and the duration of the antithrombotic effect parallels the plasma anti-Xa levels.

As yet, no satisfactory physicochemical method is available to evaluate the kinetics of the synthetic pentasaccharide. In human volunteers, plasma peak anti-Xa levels were linearly related to the dose and ranged from 0.12 to 2.1 IU/ml. The elimination $t_{1/2}$ is approximately 14 hours and is independent of the dose, but increases in the elderly because of lower renal clearance.

Safety, tolerance and effect kinetics were also investigated in a repeated rising dose study in healthy elderly volunteers for 7 days over a total daily dose range of 4000 to 12 000 IU/day administered subcutaneously once or twice daily. No major problem was observed concerning clinical tolerance. However, minor bleeding was recorded at the skin puncture and injection sites at the 2 highest doses.

14.4 DX-9065

DX-9065 is a bis-amidinoderivative with potent and highly specific factor Xa inhibitory activity.[222] After intravenous or oral administration, this compound prolongs APTT and prothrombin time, and prevents endotoxin- and thromboplastin-induced DIC in rats. This effect is independent of antithrombin III and not associated with bleeding.[223]

15. Inhibition of Factor VIII

A synthetic 12-amino acid peptide corresponding to a light-chain residue of factor VIII inhibits cleavage by thrombin of the heavy chain required for the activation of the procoagulant activity of factor VIII and also of the light chain required to dissociate factor VIII from von Willebrand factor. Tyrosine sulfation of the peptide potentiates its recognition by factor VIII.[224]

16. Vascular Anticoagulant or Endonexin V

Annexins, also called anchorins or calpactins, are proteins with 4 tandem repeating domains of approximately 70 amino acids, each exhibiting considerable homology between members. They bind with high affinity and Ca^{++}-dependently to anionic phospholipids.

An annexin with anticoagulant properties was purified from the arteries of human umbilical cord.[225] Partial purification and characterisation showed that the anticoagulant was a protein that

interfered with thrombin formation and not with thrombin action. The anticoagulant inhibited the activation of prothrombin by complete prothrombinase (factors Xa and Va, Ca^{++} and phospholipids) but not the activation by phospholipid-deficient prothrombinase. It was inferred that the anticoagulant blocked the participation of the phospholipids in the activation process.

Similar anticoagulants could be purified from bovine aortic intima and human placenta.[226] Thereafter, the protein was named vascular anticoagulant (VAC). Chemical and biochemical characterisation elucidated VAC as a nonglycosylated single chain protein of 32kD and an isoelectric pH of 4.8. Furthermore, it lacked a γ-carboxyglutamic acid residue, excluding it as a member of the vitamin K–dependent protein family.

The cDNA sequence of VAC has been determined[227,228] and expressed in *E. coli* and in yeast.[229]

The soluble recombinant protein has been purified almost to homogeneity and shown to be processed, i.e. the N-terminal methionine is cleaved off. Recombinant VAC inhibits thrombin formation but not thrombin action. Like its natural counterpart, recombinant VAC anticoagulates through a Ca^{++}-dependent high-affinity binding to negatively charged phospholipids, which function as catalytic agents in the intrinsic factor X activation and the prothrombin activation. The generation of factor Xa in thromboplastin-activated plasma is also inhibited by recombinant VAC.[226]

17. Conclusion

Considering the importance of thrombosis in cardiovascular disorders, the search for better antithrombotic strategies continues. Most of the presently available antithrombotic drugs have shortcomings, either because of limited efficacy, adverse effects and mandatory blood monitoring requirements, or because of interactions with commonly used drugs. Moreover, some are not active orally, a prerequisite for long term therapy. The combination of a potent antiaggregating agent with an anticoagulant (specific inhibitor of thrombin or factor Xa) may be a rational approach considering the interplay between platelet activation and fibrin formation in thrombogenesis. The real clinical benefit of novel antithrombotic agents and the risks of bleeding that they incur as monotherapy or in combination will only become clear when they are used in well designed large-scale clinical trials.

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Antiplatelet Agents in Stroke Prevention

Combination Therapy: Present and Future

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Key Words

Antiplatelet agents · Aspirin · Clopidogrel · Abciximab · Dipyridamole · ADP receptor · GPIIb/IIIa inhibitor

Abstract

Platelets contribute to arterial thrombosis by multiple mechanisms that promote blood clotting, favor vasoconstriction, activate the procoagulant capacity of endothelium, and stimulate inflammation. These activities are augmented by turbulent blood flow. Classic antiplatelet therapy with aspirin to prevent occlusive stroke offers significant clinical benefit (20–25% risk reduction), yet is less effective than in prevention of coronary artery occlusion (up to 50% risk reduction of myocardial infarction in unstable angina). Since aspirin's antiplatelet effects are limited to blocking a single metabolic pathway – namely inhibition of thromboxane A_2 formation –, and aspirin fails to alter platelet adhesion, other antiplatelet agents that target ADP receptors, platelet surface glycoproteins (such as the GPIIb/IIIa complex), or platelet-dependent thrombin generation offer additional clinical benefits by blocking additional separate pathways or the final common pathway of platelet activation. Combinations of antiplatelet agents, such as aspirin/dipyridamole, aspirin/clopidogrel, or aspirin/GPIIb/IIIa inhibitors, have recently been tested for improved efficacy in clinical trials. Soluble recombinant CD39, an ecto-ADPase, protects against stroke in animal models by metabolizing released ADP/ATP to antiplatelet derivatives. In general, combinations of antiplatelet agents promise greater efficacy than single drugs in preventing stroke, since interactions among different antiplatelet mechanisms can be synergistic. However, such combinations may also increase the risk of bleeding, so that precise understanding of risk/benefit ratios that address the possibility of intracranial as well as gastrointestinal bleeding will require careful monitoring in large clinical trials of patients at risk of stroke, with particular attention to the elderly.

Introduction

Pharmacologic inhibition of platelet function is an important clinical modality for preventing arterial thrombosis. Platelet activation is a key initiator of arterial vascular occlusion, whereas stasis is a dominant inciting factor for venous occlusion, as stasis favors activation of plasma procoagulants. Antiplatelet therapy for secondary prevention of acute ischemic events has long been used in clinical settings including coronary artery disease, ischemic cerebrovascular disease, and occlusive peripheral arterial disease. The use of a single antiplatelet drug, how-

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ever, provides only a moderate reduction of the thrombotic risk, the efficacy depending upon the type of vascular disease. Thus, risk reduction for acute occlusive events, as observed in multiple clinical trials, ranges from a consistent 50% decrease in myocardial infarction in patients presenting with unstable angina to about a 20% decrease in occlusive stroke in patients presenting with prior stroke or TIA. Therefore, the concept of combining two or more drugs with different modes of action for greater efficacy is attractive, provided that the combination therapy does not increase the risk of hemorrhage.

Platelet Contributions to Stroke

Activated platelets promote arterial thrombosis as a pathologic extension of the normal hemostatic response to vascular injury. Turbulent blood flow along disease-altered vascular surfaces contributes to platelet activation, as do many stroke risk factors such as elevated cholesterol, smoking, diabetes, or inflammation. Hypertension itself can incur increased platelet reactivity. Thus, risk factors associated with atherosclerotic as well as with inflammatory arterial disease contribute to enhanced platelet reactivity. Increased platelet reactivity, measured as increased aggregation in vitro in response to agonists, increased plasma or urine levels of platelet-derived mediators, membrane display of intraplatelet components, or shortened platelet survival, is often observed in patients with acute ischemic stroke [1]. Sensitive techniques such as flow-cytometric measurement of membrane display of platelet activation markers, or measurement of released platelet-specific metabolites such as 11-dehydrothromboxane B_2 have recently confirmed that platelet activation is enhanced in patients presenting with acute cerebral ischemia [2, 3], whereas prior techniques had been either too insensitive or too nonspecific for unequivocal demonstration of platelet hyperactivity. Part of the observed platelet hyperactivity reflects an acute inflammatory state induced by damage to cerebral tissues. However, following cerebral ischemia or intracranial hemorrhage platelets display chronic activation that is not detected following cerebral embolic events [4]. For example, increased platelet aggregability has been shown to persist in 60% of the patients at 3 and 9 months after stroke, i.e., well after acute inflammatory responses had resolved, and was correlated with poorer outcome. However, such chronic platelet hyperactivity could be suppressed by aspirin treatment [5, 6]. Furthermore, increased circulating levels of procoagulant proteins such as fibrinogen, factor VIII, and von Willebrand factor and increased levels of inflammatory markers such as C-reactive protein and white blood cell count are also associated with ischemic stroke [6–8]. Recent infection may also contribute to the risk of cerebral ischemic events [9]. The seasonal rise in stroke observed in the elderly during the winter may reflect an increase in infections resulting in a transient inflammatory state [10]. An important link between inflammation and increased stroke risk may be the potentiation by cytokines released during inflammation of several processes that favor vascular occlusion, namely increased platelet reactivity, activation of vascular endothelium, and leukocyte-vascular interactions.

Platelet Functions Contributing to Stroke Risk

Platelet activation initiated by vascular injury consists of a rapid series of interactive steps that culminate in a hemostatic primary platelet plug which functions to prevent blood loss at the injury site without occluding blood flow. These normal activation steps include (1) the adhesion of individual platelets to a damaged vessel wall; (2) their spreading across the injured surface; (3) aggregation of additional platelets upon the initial monolayer of spread platelets; (4) release of procoagulants, platelet-activating substances (e.g., ADP or thromboxane), enzymes, growth factors, and inflammatory mediators from individual activated platelets, and (5) the acceleration of thrombin generation localized on the surface of the activated platelets. In contrast, platelet activation that is inappropriate or excessive in deploying this series of steps leads to thrombosis, i.e., the formation of a clot that occludes blood flow and, therefore, produces tissue ischemia.

Three main physiologic pathways initiated by different ligands contribute to platelet activation: the first pathway activated via ADP, the second activated via arachidonate and its metabolite thromboxane A_2, and the third pathway activated via thrombin. Each pathway is mediated by different classes of membrane receptors and independent signalling pathways (table 1). All three pathways converge in a final common mechanism, the activation of the GPIIb/IIIa receptor complex on the platelet surface, permitting the binding of fibrinogen and leading to consequent platelet-platelet interactions, the formation of platelet aggregates, and the release of stored mediators that promote hemostasis, inflammation, and wound healing. Moreover, the activated platelet membrane offers a

Table 1. Platelet functions, activation pathways, and therapeutic modulators

Platelet function	*Physiologic pathway*	*Modulating drug*
Adhesion to vessel wall	GPIb, von Willebrand factor	GPIb inhibitors Dipyridamole
Fibrinogen binding	GPIIb/IIIa	Abciximab, fibans
Aggregation	GPIIb/IIIa, ADP, TXA_2 Thrombin	Aspirin, NSAIDs Thienopyridines Iloprost Abciximab, fibans NO donors soluble CD39
Release reaction (vasoactive factors, ADP, Ca^{2+}, growth factors, adhesive proteins procoagulants, serotonin)	ADP, GPIIb/IIIa, TXA_2	Aspirin, NSAIDs Abciximab, fibans soluble CD39
	Thrombin	Anticoagulants, direct antithrombins
Procoagulant activity	GPIIb/IIIa, ADP, TXA_2	Anticoagulants Combined therapy with ASA/GPIIb/IIIa inhibitors Dipyridamole

specialized catalytic surface for the assembly of the prothrombinase complex in the coagulation cascade and therefore localizes marked acceleration (100,000-fold) of the rate of formation of thrombin, which in turn catalyzes rapid localized clot formation and strongly activates additional platelets.

Antiplatelet therapy blocks one or more of these receptor-mediated pathways. Antiplatelet therapies mainly directed against platelet aggregation and mediator release tend to block platelet-initiated thrombosis without abolishing hemostasis, whereas therapies that inhibit the initial steps of platelet adhesion can be expected to block hemostatic platelet responses as well, incurring greater bleeding risk. The capacity of platelets to accelerate thrombin formation is not decreased by most individual antiplatelet agents, except direct inhibitors of thrombin or factor Xa, but may be achieved by combinations of antiplatelet drugs.

These different types of antiplatelet activity are differently affected by currently used antiplatelet drugs. Aspirin, by selectively inhibiting platelet cyclooxygenase and, therefore, abolishing thromboxane A_2 formation, partially blocks platelet aggregation and platelet release, without affecting platelet adhesion or thrombin generation. Clopidogrel, an ADP receptor antagonist, partially blocks activation of platelet ADP receptors of the $PY2_{ac}$ type that participate in platelet aggregation induced by a variety of agonists, without affecting thromboxane formation.

Dipyridamole, a phosphodiesterase inhibitor, permits platelet cAMP to remain elevated once it is raised; high cAMP levels make platelets unreactive; dipyridamole also decreases platelet adhesion and has been reported to help prolong abnormally short platelet survival. Abciximab, a humanized mouse monoclonal antibody, blocks activation of platelet glycoprotein IIbIIIa receptors, inhibiting all pathways of platelet response and producing a temporary thrombasthenic state in which platelets fail to aggregate in response to normal agonists.

Aspirin and Other Nonsteroidal Anti-Inflammatory Drugs

Aspirin rapidly and irreversibly acetylates platelet cyclooxygenase, destroying its capacity to convert arachidonic acid to prostaglandin G_2, thus preventing thromboxane A_2 generation and platelet activation via thromboxane A_2. As a result, both platelet aggregation and platelet release are decreased, but neither platelet response to thrombin nor platelet adhesion is altered. Since circulating platelets do not synthesize new cyclooxygenase, the effect of a brief exposure to aspirin lasts for the life span of the aspirinated platelet: 7–10 days.

The use of aspirin for secondary prevention of stroke closely followed its use in prevention of occlusive cardiac events, based on the assumption that aspirin would have

similar efficacy in different regions of the arterial circulation. While in general terms, this assumption has proved correct, the extent of protection against stroke has been considerably less than the protection against myocardial infarction. The difference in efficacy probably relates to the greater role of platelet-rich thrombi in coronary arterial ischemia/occlusion than in cerebral ischemia, possibly stemming from differences in rates of plaque rupture. The optimum dose of aspirin for antiplatelet effects has been a controversial subject, with the FDA recommendation in the United States being 80–325 mg of aspirin per day. Numerous studies have demonstrated that, at doses between 30 and 1,500 mg/day, aspirin reduces the risk of a cerebral ischemic event by 16–25% without a clear dose-related effect within that dosing range [11]. In completed acute stroke, very large recent trials have confirmed a small but clearcut benefit of early use of aspirin [12, 13]. Doses higher than 325 mg/day have been reported to result in decreased cerebral blood flow [14] and increased risk of intracranial bleeding [15]. Patients with 'aspirin resistance' often have additional factors that increase platelet sensitivity to agonists, such as catecholamine-induced decreases in cAMP levels that oppose the antiplatelet effects of aspirin [16]. Limitations of aspirin use include allergy to the drug and gastric irritation and bleeding. Bleeding complications increase in a dose-dependent manner, but even low doses can cause bleeding in susceptible individuals, particularly the elderly.

Aspirin's superiority over other nonsteroidal anti-inflammatory drugs relates to its irreversible inactivation of platelet cyclooxygenase. This unique feature permits a prolonged antiplatelet effect with a single daily dose, even though in vivo aspirin is rapidly deacetylated to salicylate which has little to no antiplatelet activity. Other nonsteroidal anti-inflammatory drugs reversibly inhibit platelet cyclooxygenase, so that therapeutic efficacy requires maintenance of high plasma drug levels and multiple daily dosing that is difficult to maintain long term. Since platelets contain only cyclooxygenase-1, the new specific cyclooxygenase-2 inhibitors such as celecoxib and rofecoxib do not alter platelet functions and are not effective for antiplatelet therapy.

Thienopyridine Drugs: Clopidogrel and Ticlopidine

Thienopyridines such as clopidogrel and ticlopidine specifically block the binding of ADP to PY_{AC}-type purinergic receptors on platelets and therefore inhibit ADP-mediated platelet functions, including the activation of GPIIb/IIIa, the fibrinogen receptor, to its high-affinity form [17, 18]. These prodrugs are inactive until converted in the liver to active metabolites; the parent drug has no effect on platelets in vitro. Clopidogrel is converted to its active metabolite on first pass through the liver, but ticlopidine circulates. Full clinical effects of both drugs are achieved only after several days, and the antiplatelet effects last up to 1 week after the drug is stopped. During therapy, the bleeding time is markedly prolonged in a dose-dependent manner. Clopidogrel becomes effective more rapidly than ticlopidine, and a 'loading dose' of 300 mg permits antiplatelet effects within a few hours. Thienopyridines also modulate vasoconstriction by several mediators such as serotonin, endothelin, or thromboxane, possibly acting through ADP receptors on the vascular wall [19].

Ticlopidine, marketed over 10 years ago, had been shown to be more effective (by 12%) than aspirin in secondary prevention of cerebral ischemia at a dose of 250 mg twice daily [17]. Ticlopidine also became widely used in combination with aspirin and heparin to prevent thrombosis after cardiac interventions such as coronary artery stenting and angioplasty. However, ticlopidine has a high rate of adverse hematologic side effects that include unpredictable severe neutropenia and thrombocytopenia (in up to 2.4% of the recipients) and, in particular, thrombotic thrombocytopenic purpura (TTP; estimated to occur in 1 in 1,500 to 1 in 4,000 recipients of the drug) [20]. These adverse effects have resulted in its replacement by clopidogrel, a closely related compound that was first marketed in 1998.

In the CAPRIE study [21] of 19,185 subjects, clopidogrel 75 mg once daily was compared to 325 mg aspirin for secondary prevention of stroke, myocardial infarction, or occlusive peripheral vascular disease. Clopidogrel provided an 8.7% increase in risk reduction over aspirin for the combined end point of ischemic stroke/myocardial infarction/vascular death. The benefit of clopidogrel is consistent for all the vascular disease related end points, the benefit being greatest for prevention of myocardial infarction (19.2%). It has also been recently shown that clopidogrel reduces the risk of hospitalization for ischemic events or bleeding [21a]. The availability of clopidogrel has led to a major shift away from ticlopidine use in cardiac interventions, since the safety profile of clopidogrel during the CAPRIE study appeared to be distinctly better than that of ticlopidine: no excess of leukopenia or thrombocytopenia, less diarrhea and rash, and no TTP [22]. However, during recent postmarketing surveillance,

11 cases of suspected TTP were reported among 3 million patients receiving clopidogrel [23]. The background incidence of TTP in the general population is estimated as 4 cases per million. The causal relationship with clopidogrel has not been formally established, but in 10/11 of the cases TTP developed during the first 14 days of receiving the drug. All but 1 case responded to vigorous plasmapheresis, although several patients relapsed and required retreatment.

Combined Aspirin and Dipyridamole

Because the antiplatelet effects of aspirin and dipyridamole are different and complementary, clinical trials of this combination were performed in the 1970s and 1980s, but failed to demonstrate enhanced efficacy over aspirin monotherapy. Problems with dipyridamole therapy have included highly variable gastrointestinal absorption of the drug, a frequent incidence of headache, plus poor compliance because of the need for four daily doses. Dipyridamole then fell into disuse for stroke prevention until data became available from the ESPS 2 trial comparing twice-daily dosing of low-dose aspirin, a slow-release dipyridamole formulation, or the combination versus placebo [24]. In this secondary prevention trial involving over 6,000 patients, each drug alone showed 16% risk reduction over placebo, but the combination showed a 37% risk reduction, a beneficial effect in stroke prevention distinctly greater than that achieved for aspirin alone in any previous randomized clinical trial. The effects of the two drugs are additive. The extended-release dipyridamole used in the trial was formulated to improve its absorption from the gastrointestinal tract. The ESPS 2 data confirmed data obtained in an earlier, smaller trial, ESPS 1. A combined tablet incorporating both drugs is now available both in the USA (where FDA approval was obtained in 1999) and in Europe; each tablet contains 25 mg of aspirin and 200 mg of dipyridamole for twice-daily dosing. Intolerance of dipyridamole-induced headache appears to deter use in some patients.

Dipyridamole is known to inhibit superoxide ion generation and to decrease tissue factor expression by leukocytes [25]; since activated platelets promote leukocyte and endothelial activation, which further contribute to cerebral ischemia, these additional effects of dipyridamole complement its direct antiplatelet effects to prevent stroke.

Combined Aspirin and Clopidogrel

Since aspirin and clopidogrel affect platelet function by different mechanisms that block separate pathways, using the combination to increase antiplatelet efficacy appears logical, provided that bleeding complications do not increase. This drug combination is rapidly replacing aspirin/ticlopidine as standard practice to prevent coronary artery reocclusion after cardiac interventions such as coronary angioplasty and stenting. Ticlopidine plus aspirin was previously used extensively for this indication. In one study [26] ticlopidine/aspirin resulted in less stent thrombosis (0.5%) as compared with aspirin alone (3.6%) or aspirin/warfarin (2.7%), although the rate of bleeding was slightly increased. In a randomized study of 1,020 patients undergoing coronary artery stents [27], the use of clopidogrel/aspirin resulted in primary event rates of cardiac deaths, need for urgent revascularization, or myocardial infarction similar to the use of ticlopidine/aspirin (1.3 vs. 0.9% at 4 weeks; $p = 0.76$), while secondary events of noncardiac death, stroke, or hemorrhage were less with clopidogrel/aspirin (4.6 vs. 9.1%, $p = 0.01$), and the use of an initial loading dose of clopidogrel shortened the time to effective activity. These results confirmed those obtained in other studies which compared patients who had received ticlopidine/aspirin after stenting with patients who received clopidogrel/aspirin at a later time [27a, 28, 29]. The combination of clopidogrel plus aspirin is being studied in several ongoing trials of high-risk patients: CURE in unstable angina; MATCH in patients with TIA/stroke with at least one additional risk factor; CREDO in coronary stenting.

Combined Aspirin and Warfarin

Long-term oral anticoagulation protects against stroke in patients with atrial fibrillation, but with a considerable risk of bleeding, particularly in the elderly and in those with full-dose anticoagulation. The possibility of combining antiplatelet and anticoagulation therapy using lower doses of each has been recently explored. Several clinical trials have compared low-dose warfarin (often 1 mg/day or maintaining INR at about 1.5) plus aspirin with full, adjusted-dose warfarin or aspirin alone for prevention of stroke in atrial fibrillation [30, 31] or for secondary prevention of myocardial infarction or stroke in patients with atherosclerosis. No advantage in efficacy or safety has been observed for the aspirin/low-dose warfarin therapy, nor was the activation of the coagulation system de-

creased; moreover, the combination of low-dose aspirin/full-dose warfarin produced excess bleeding, so, in general, this approach is no longer being pursued.

GPIIb/IIIa Antagonists

Abciximab is a humanized monoclonal antibody that binds to the GPIIb/IIIa complex on platelets, blocking the final common pathway of platelet activation [32]. Because abciximab induces a profound impairment of platelet function, similar to a temporary state of thrombasthenia, the rate of major bleeding is high. Up to the present time, it has been used mainly in cardiac patients, on a short-term basis, as it is immunogenic. Abciximab is used in combination with aspirin and heparin after invasive coronary artery interventions to decrease occlusive cardiac events or need for revascularization. It has not been effective when used alone. Other parenteral, small molecule, nonantibody GPIIb/IIIa antagonists, tirofiban and eptifibatide, also improve the outcome after coronary artery interventions and may have lesser hemorrhagic side effects [33, 34]. However, attempts to employ oral GPIIb/IIIa antagonists combined with aspirin in long-term prevention of ischemic cardiac events after percutaneous coronary revascularization have been unsuccessful in humans. Numerous trials have been stopped and abandoned because of increased thrombosis (e.g. 35]. Therefore, no studies have been undertaken to date with GPIIb/IIIa inhibitors for secondary prevention of stroke.

Abciximab to Treat Acute Occlusive Stroke

However, based on the success of parenteral GPIIb/IIIa inhibition short-term to limit acute arterial occlusive events in patients undergoing coronary angioplasty, stenting, and thrombolysis, studies are under way to examine abciximab as treatment of acute ischemic stroke. The rationale is based on the observation that abciximab, aspirin, and adjusted-dose heparin result in reopening of up to 50% of occluded coronary arteries in patients with acute myocardial infarction without serious intracranial bleeding [32]. Animal studies also suggested that GPIIb/IIIa antagonists restore cerebral arterial blood flow after occlusion [36, 37]. As a first step, a safety study has recently been completed, suggesting that using abciximab during the first 24 h after stroke onset is feasible without incurring major intracranial hemorrhage (asymptomatic parenchymal hemorrhage detected in only 4/54 patients receiving abciximab and in 1/20 receiving placebo) [38]. The study was not empowered to examine efficacy.

Future Antiplatelet Approaches under Development

ADP is an important agonist for hemostasis and thrombosis via its effects on the three types of platelet purinergic receptor, P2X1, P2Y1, and $P2_{AC}$ (also called P_{2T}). P2X1 is a ligand-gated ion channel responsible for calcium influx; P2Y1 mobilizes ionized calcium from internal platelet stores and activates protein kinase C, whereas $P2_{AC}$ is coupled to adenylate cyclase inhibition and is required for full platelet aggregation. Normal platelet aggregation requires activation of both P2Y1 and $P2_{AC}$. Desensitization of platelet response to ADP involves the internalization of P2Y1 receptors, suggesting a potential target for antiplatelet therapy. In this regard, natural examples of altered hemostasis resulting from mutations in platelet ADP receptors are of interest. P2Y1 receptor null mice have defective platelet aggregation and resistance to thrombosis [39]. Rare patients have been recently described whose platelets fail to respond to ADP, but it is not certain that absence of the P2Y1 receptor is responsible. Moreover, a child with a severe bleeding disorder has been shown to carry a dominant-negative mutation of the gene for the P2X1 receptor. As noted above, clopidogrel is an antagonist of the $P2_{AC}$ rather than the P2Y1 receptor, and at clinical doses partially inhibits that receptor.

Specific nucleotide analog inhibitors of the $P2_{AC}$ or P2Y1 receptors are under development as antiplatelet agents. AR-C69931MX [2-trifluoropropylthio, Nn-(2-methylthio)-β,γ-dichloromethylene ATP], a nucleotide analog, is a potent inhibitor of ADP-mediated platelet aggregation in vitro at subnanomolar concentrations both in washed platelets and in whole blood. In the Folt model of platelet-dependent arterial thrombosis in dogs, intravenous AR-C69931MX was more potent than GPIIb/IIIa inhibitors in abolishing thrombus-related cyclic flow variations. In addition, AR-C did not prolong the bleeding time at antithrombotic doses, and its effect was rapidly reversible on stopping the infusion. Moreover, when administered to patients with unstable angina or non-Q-wave infarction, AR-C produced greater inhibition of ADP-induced platelet aggregation and release than did clopidogrel [40]. Used together, the two drugs had additive effects. Basic studies of PY21 inhibitors have also shown that platelet procoagulant activity can be decreased by this modality.

Soluble CD39 as a Novel Antiplatelet Agent to Prevent Stroke

When activated platelets come into proximity with endothelial cells in vitro, they lose responsiveness to aggregating agonists. Even after blockade of both endothelial prostacyclin and nitric oxide synthesis, endothelial cells exert antiplatelet activity, suggesting an additional modality by which the vessel wall controls platelet activation. CD39, an ecto-ADPase that is localized on the lumenal side of the endothelial cell plasma membrane, is responsible for this antiplatelet activity [41]. CD39 metabolizes ATP and ADP released by platelets to AMP which is in turn converted to adenosine by the endothelial enzyme ecto-5′-nucleotidase. Adenosine inhibits platelet activation, is vasodilatory, and suppresses tissue factor induction, all antithrombotic effects. A transgenic mouse lacking the enzymatic portion of the CD39 molecule has a normal phenotype, but responds to transient carotid artery injury with larger cerebral infarcts and lower postischemic perfusion as compared with CD39-normal animals [42]. This indicates that endogenous CD39 is thromboprotective. A soluble recombinant CD39 infused either before or after the carotid injury in this stroke model decreases cerebral infarct size and the neurologic deficit in the CD39 null animals without incurring bleeding [42]. In contrast, in CD39 null mice, aspirin failed to decrease infarct size, but increased intracerebral hemorrhage. Soluble CD39 also inhibited platelet activation in vivo in mice and in pigs [43]. It has a prolonged effect lasting days. Thus, a soluble recombinant form of CD39, a normally expressed endothelial enzyme, has promise as a thromboprotective agent in acute cerebral ischemia for inhibition of thrombosis without increasing intracranial hemorrhage.

Conclusion

Current antiplatelet agents address a variety of platelet functions that contribute to thrombosis. Each has modest to moderate efficacy at doses that do not impair hemostasis. Their effects are mainly additive when used in combination, but certain combinations can decrease the key platelet activity of accelerating thrombin formation without recourse to anticoagulation. Secondary effects on leukocyte or endothelial activation may also promote efficacy of antiplatelet agents in preventing arterial thrombosis. Recently developed aspirin/dipyridamole combination therapy appears to have an advantage for secondary prevention of stroke, probably on the basis of more effective delivery of dipyridamole. Combinations of aspirin/clopidogrel are currently in large-scale clinical trials, with prediction of additive to synergistic effects. To date, long-term administration of GPIIb/IIIa inhibitors has not shown efficacy, although short-term use in cardiac settings is valuable; evaluation of efficacy and safety in treatment of acute stroke is beginning. In the future, modulation of the ADP receptor and/or conversion of released ADP to endogenous antiplatelet derivatives may represent promising new modalities of antiplatelet therapy.

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Administration of a Potent Antagonist of Protease-Activated Receptor-1 (PAR-1) Attenuates Vascular Restenosis Following Balloon Angioplasty in Rats

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ABSTRACT

Human platelets possess two distinct thrombin-activated receptors, PAR-1 (protease-activated receptor-1) and PAR-4, whereas human vascular smooth muscle cells possess only PAR-1. Although such thrombin receptors have been studied extensively in vitro, their physiological roles are still rather ill-defined. We have now employed a potent, selective PAR-1 antagonist, RWJ-58259, to probe the in vivo significance of PAR-1 in thrombosis and vascular injury. RWJ-58259 was examined in two thrombosis models in guinea pigs: the arteriovenous (A-V) shunt assay (monitoring thrombus weight) and the Rose Bengal intravascular photoactivation assay (monitoring time to occlusion). Administration of RWJ-58259 (10 mg/kg, total i.v. dose) did not inhibit thrombus formation in either thrombosis model, although local, intrashunt delivery in the A-V shunt model did elicit a modest antithrombotic effect (thrombus weight reduction from 35 ± 2 to 24 ± 4 mg). These results are consistent with the presence of more than one thrombin-sensitive receptor on guinea pig platelets, in analogy with human platelets. Indeed, we were able to establish that guinea pig platelets express three thrombin receptors, PAR-1, PAR-3, and PAR-4. We also examined RWJ-58259 in a vascular restenosis model involving balloon angioplasty in rats. Perivascular administration of RWJ-58259 (10 mg) significantly reduced neointimal thickness (77 ± 5 μm to 45 ± 5 μm, $P < 0.05$), clearly demonstrating an important role for PAR-1 in vascular injury. From these results, it is evident that a PAR-1 antagonist is not especially effective for treating platelet-dependent thrombosis; however, it could well be beneficial for treating restenosis attendant to arterial injury.

α-Thrombin is a powerful agonist for a variety of cellular responses, and these actions are mediated by a special type of G protein-coupled transmembrane receptor known as a protease-activated receptor (PAR). Important biological effects of thrombin are mediated by such PARs in platelets, fibroblasts, monocytes, neutrophils, osteoblast-like cells, smooth muscle cells, nerve cells, and endothelial cells (Coughlin, 1994; Dennington and Berndt, 1994; Ogletree et al., 1994; Van Obberghen-Schilling et al., 1995). Perhaps the best-characterized receptor function of thrombin is the activation of platelets, which is a crucial process in thrombosis and hemostasis. Thrombin is the most potent stimulator known of platelet aggregation and degranulation, and it may also be the most significant mediator of platelet recruitment during arterial thrombus formation. Thrombin-induced aggregation of human platelets is mediated by two PARs, PAR-1 and PAR-4 (Kahn et al., 1999), whereas PAR-1 is not relevant to thrombin-induced aggregation of rat or mouse platelets (Connolly et al., 1994; Derian et al., 1995). This species dependence makes it problematic to derive a good understanding of the in vivo physiology associated with different PARs.

The role of thrombin receptor activation in thrombosis and hemostasis could be demonstrated more clearly with specific pharmacological agents that can interrupt receptor function. Recently, we identified a series of potent, indole-based peptide-mimetic PAR-1 antagonists, represented by RWJ-56110, the biological function of which was characterized in vitro (Andrade-Gordon et al., 1999). This antagonist was very selective in blocking the actions of PAR-1 over the actions of PAR-2, PAR-3, or PAR-4. Interestingly, we found that the inhibitory effect of RWJ-56110 in thrombin-induced human platelet aggregation is attenuated at high enzyme levels (e.g., 8 nM), consistent with PAR-4 playing a role in thrombin signaling (at the elevated concentrations). This scenario raises a serious question about the ability of a PAR-1 antagonist to serve effectively as an antithrombotic agent, an issue of keen interest since the identification of PAR-1 in 1991 (Vu et al., 1991). We report herein the first in vivo investigation

ABBREVIATIONS: PAR, protease-activated receptor; RT, reverse transcriptase; PCR, polymerase chain reaction; bp, base pairs.

of the antithrombotic effects of a potent peptide-mimetic PAR-1 antagonist, RWJ-58259, by using two standard animal models. Since thrombin is implicated in the proliferative and inflammatory events associated with restenosis, we have also investigated the effects of RWJ-58259 in a rat model of vascular injury. Our results clearly suggest that a PAR-1 antagonist has the potential for therapeutic utility in restenosis following balloon angioplasty.

Experimental Procedures

Materials. RWJ-58259 was synthesized in our laboratories, purified by flash-column chromatography, and isolated as a dihydrochloride dihydrate (off-white powder). Details on the synthesis and isolation will be published separately. The structure of RWJ-58259 was confirmed by NMR spectroscopy and mass spectrometry; the purity was established by elemental microanalysis and reverse-phase high-pressure liquid chromatography.

Platelet Aggregation. Human platelet-rich plasma concentrate containing the anticoagulant acid-citrate dextrose (Biological Specialty Corp., Colmar, PA) was gel-filtered (Sepharose 2B, Amersham Pharmacia Biotech Inc., Piscataway, NJ) in Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.76 mM Na_2HPO_4, 5.5 mM dextrose, 5.0 mM Hepes, and 2 mg/ml bovine serum albumin, pH 7.4). Gel-filtered platelets were diluted with Tyrode's buffer (143,000 platelets/μl per well), compound solution in buffer, and 2 mM $CaCl_2$ in a 96-well microtiter plate. All fresh blood samples were obtained using sodium citrate (0.38% final concentration) as the anticoagulant. For platelet-rich plasma studies, human blood was obtained by venipuncture from healthy volunteers who were drug free for a minimum of 10 days. Guinea pigs (Hartley; Covance Inc., Denver, PA) or rats (Sprague-Dawley, Charles River, Raleigh, NC) were anesthetized and blood drawn via an intra-arterial catheter. Platelet-rich plasma was prepared by centrifugation at 200*g* for 10 min. Platelet-rich plasma aggregation was performed in the presence of 4 mM H-Gly-Pro-Arg-Pro-NH_2 to inhibit fibrin polymerization. Platelet aggregation was initiated by addition of an agonist shown to achieve 80% aggregation. The α-thrombin concentrations for gel-filtered platelet and platelet-rich plasma aggregation studies were 0.15 and 7.5 nM, respectively. The SFLLRN-NH_2 concentration used was 2 μM. The assay plate was gently mixed constantly. Aggregation was monitored at 0 and 5 min after agonist addition in a microplate reader by optical density at 650 nm (Molecular Devices, Sunnyvale, CA). Aggregation was calculated as the decrease in optical density between the two measurements. All samples were tested in duplicate wells on the same plate.

Cell Cultures. Human aortic smooth muscle cells and growth media were obtained from Cascade Biologics (Portland, OR). Rat aortic smooth muscle cells were obtained from Cell Applications (San Diego, CA) and were cultured as described (Owens et al., 1986).

Calcium Mobilization. Intracellular calcium mobilization was measured using a fluorescence technique. Rat aortic smooth muscle cells in 96-well microtiter plates were loaded with 5 μM fluo-3-AM (Molecular Probes, Eugene, OR) for 90 min. Plates were washed five times to remove unincorporated dye. Subsequent steps were performed using a fluorometric imaging plate reader (FLIPR, Molecular Devices). Test compounds were added and cells were monitored for 5 min to detect any inherent agonist activity. Thrombin (2 nM) was added and the fluorescence signal was recorded for 3 min. Net peak calcium, expressed in arbitrary fluorescence units, was measured automatically.

DNA Synthesis. Cell proliferation was measured by [^{14}C]thymidine incorporation. Rat aortic smooth muscle cells were plated on Cytostar scintillating plates (Amersham). After 4 days of growth, cells were depleted of serum for 4 days (Owens et al., 1986). Thrombin (0.8 nM) was added in fresh media and cells were incubated for 24 h. [^{14}C]Thymidine was added and incubation was continued for 24 h. [^{14}C]Thymidine incorporation was measured in a Wallac MicroBeta counter (Wallac, Gaithersburg, MD) without additional processing steps.

Interleukin-6 Assay. For measurement of interleukin-6 release, human aortic smooth muscle cells plated on 96-well microtiter plates were quiesced in Medium 231 (Cascade) containing 0.5% fetal bovine serum for 3 days. Thrombin (2 nM) was added in fresh serum-free Medium 231 and supernatants were collected after overnight incubation. Samples were analyzed by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

PCR Analysis for PAR-1 and PAR-4. Total RNA was isolated from guinea pig washed platelets using Trizol Reagent (Life Technologies, Grand Island, NY). For conversion of RNA to first-strand cDNA, samples were incubated with random primers in the presence or absence (minus RT for negative controls) of Superscript II reverse transcriptase (Life Technologies) according to the manufacturer's recommendations. PCR reactions were carried out on ca. 50 ng of cDNA, or equivalent amounts of RNA in the RT reactions, using the Advantage-GC cDNA polymerase mix (CLONTECH, Palo Alto, CA). Primers to generate and detect the respective guinea pig PAR amplicons were designed using the nucleic acid alignments of the known species for PAR-1 and PAR-3. However, numerous attempts to use this strategy to detect guinea pig PAR-4 were unsuccessful. Thus, the sequences used to amplify and detect the guinea pig PAR-4 PCR product were designed from the partial sequence analysis of the guinea pig PAR-4 gene (manuscript in preparation). The sense and antisense primers used for the amplification of PAR sequences were: PANP1-U, 5′-CATAAGCATTGACCGGTTCCTGGC-3′; PANP1-L, 5′-CAAAGCAGACGATGAAGATGCAGA-3′; PANP3-U, 5′-CAATGGCAACAACTGGGTATTTGG-3′; PANP3-L, 5′-AAAATCACAAGGATGAGGAG-3′; GPPANP4-U, 5′-TGGCCGTGGGGCTGCCGGCC-AATG-3′; and GPPANP4-L, 5′-GTCAACACAGCTGTTGAGGGTGCT-3′.

Reactions were conducted at a volume of 50 μl and at 25 cycles of 94°C for 30 s, 60.1°C for 30 s, and 68°C for 48 s for PAR-1; 20 cycles of 94°C for 30 s, 54.4°C for 30 s, 68°C for 56 s for PAR-3; and 28 cycles of 94°C for 30 s, 63.5°C for 30 s, and 68°C for 90 s for PAR-4. The products of each reaction (5.0 μl for PARs 1 and 3, and 50.0 μl for PAR-4) were electrophoresed through 2% agarose gels and transferred to Hybond N+ membranes (Amersham). The appropriate oligonucleotide primer probes, corresponding to nested sequences within the respective PAR PCR product, were digoxigenin-labeled, hybridized, and detected using the Genius nucleic acid detection system (Roche Molecular Biochemicals, Indianapolis, IN). The sequences used for these nested primer probes were: PANP1PP-L, 5′-CCAGAGTGCGCCAGGACAGGGACTGGATGGGGTACACCAC-3′ for PAR-1; PANP3PP-L#3, 5′-TCCTCACTTGCATGGGCATCAACCGCTACCTGGCCAC-3′ for PAR-3; and GPPANP4PP-L, 5′-CGGGCACGCAGGGGGTGCACCAGCGCCAGGTAGCGGTCCAGGCTGA-3′ for PAR-4.

Animal Models. All procedures involving the use of animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and the Animal Care and Use Committee, The R. W. Johnson Pharmaceutical Research Institute, Spring House, PA.

Ex Vivo Platelet Aggregation. RWJ-58259 was administered i.v. to anesthetized guinea pigs at the indicated doses as a 5 min infusion. Blood was withdrawn 5 min after dosing. Inhibition of thrombin or SFLLRN-induced platelet aggregation was assessed using platelet-rich plasma.

Guinea Pig Arteriovenous Shunt Thrombosis Model. Adult male guinea pigs (Hartley, 600–750 g) were anesthetized with a ketamine hydrochloride/xylazine hydrochloride solution i.m. The left jugular vein was cannulated (PE-50) for drug administration. The left carotid artery and right jugular vein were cannulated with silicon treated (Sigmacote, Sigma Chemical, St Louis, MO), saline-filled polyethylene tubing (PE-60) and connected with a 6-cm section of silicon-treated tubing (PE-190) to form an extracorporeal arteriovenous shunt. Shunt patency was monitored using a Doppler flow

system (model VF-1, Crystal Biotech Inc., Hopkinton, MA) and flow probe (1.0 mm, Titronics, Iowa City, IA) placed proximal to the shunt.

On completion of a 15-min postsurgical stabilization period, RWJ-58259 was administered intravenously as a loading-plus-maintenance infusion or directly into the shunt as a constant infusion. An occlusive thrombus was formed by the placement of a thrombogenic surface (#50 cotton thread, 6 cm in length) into the extracorporeal shunt. After 15 min exposure to flowing blood, the cotton thread was carefully removed and thrombus weight was calculated by subtracting the weight of the thread (3 mg) prior to placement from the total wet weight of the thread upon removal from the shunt. Arterial blood was withdrawn immediately at the conclusion of the study to assess ex vivo platelet function and coagulation.

Platelet count determinations were performed using a Sysmex K1000 differential cell counter (Sysmex Corporation, Kobe, Japan). Platelet-rich plasma aggregation induced by α-thrombin (35 nM) or SFLLRN-NH_2 (50 μM) was measured using an aggregation profiler (Bio/Data model PAP-4, Bio/Data Corp., Horsham, PA). Activated clotting time was determined using a whole-blood microcoagulation analyzer (Hemochron Jr., International Technidyne Corp., Edison, NJ). Template bleeding-time measurements were performed by the toenail-clip method, monitoring the time to clot formation.

RWJ-58259 was intravenously administered as a 5 mg/kg loading dose (over 5 or 10 min) with a subsequent 5 mg/kg maintenance infusion (over 20 min) for a total cumulative dose of 10 mg/kg. Inogatran (synthesized at the R. W. Johnson Pharmaceutical Research Institute) was administered as a 0.7 mg/kg loading dose (over 1 min) with a subsequent 0.3 mg/kg maintenance infusion (over 19 min) for a total cumulative dose of 1 mg/kg. Aspirin was administered at 100 mg/kg (over 2 min) and the shunt protocol was started 5 min later. This dose of aspirin was chosen based on previous studies whereby lower doses of aspirin had been ineffective in reducing thrombus weight. In a separate series of experiments, RWJ-58259 was administered directly into the shunt at a constant infusion of 0.1 or 0.3 mg/kg/min (over 20 min) for a total cumulative dose of 2 or 6 mg/kg, respectively. Inogatran was administered directly into the shunt at a constant infusion of 0.01 mg/kg/min (over 20 min) for a total cumulative dose of 0.2 mg/kg.

Intravascular Photoactivation Model. Male guinea pigs (Hartley, 375–700 g) were anesthetized with ketamine/xylazine (90/12 mg/kg, i.m.) and the right carotid artery gently isolated from the surrounding connective tissue. A 1-mm ultrasonic Doppler flow probe was secured around the artery proximal to the occlusion area and flow was continuously measured. Rose Bengal (Sigma), a photoactive dye, was dissolved in saline and infused i.v. at 20 mg/kg over 10 min. A green, heat-filtered xenon light source, positioned 0.5 cm from the artery to illuminate a 1-cm length of the vessel, was turned on 5 min before Rose Bengal infusion and remained on for 15 min. Arterial flow was monitored for a total of 30 min following the start of the Rose Bengal infusion. RWJ-58259 was administered at a total dose of 10 mg/kg, i.v., split into a 5 mg/kg infusion for 10 min prior to Rose Bengal and 5 mg/kg infusion starting after the conclusion of the Rose Bengal infusion for the remaining 20 min of the 30-min observation period. Recombinant hirudin (Hoechst Marion Roussel, Kansas City, MO) was infused at either 1 or 3 mg/kg i.v. for 10 min prior to the Rose Bengal infusion. RWJ-58259 was dissolved in 5% dextrose and r-hirudin was dissolved in saline. In a separate group of RWJ-58259-treated guinea pigs, not exposed to Rose Bengal or light, the animals were exsanguinated, platelet-rich plasma was prepared, and ex vivo platelet aggregation to α-thrombin and SFLLRN-NH_2 was measured.

Rat Restenosis Model. Vascular injury was induced by balloon-catheter inflation of the rat common carotid artery. A 2F embolectomy catheter was inserted via the external carotid into the left common carotid of male Sprague-Dawley rats (350–450 g) anesthetized with ketamine/xylazine (75/5 mg/kg, i.m.). The balloon tip was advanced to the aorta, inflated to 35 psi, and slowly withdrawn a total of three times. RWJ-58259 (1, 5, or 10 mg) was suspended in 150 μl of a polymer gel consisting of 50% capryolate and 50% glycolate and applied to the adventitia of the left common carotid. This polymer was shown not to affect the vascular injury response in this model. Perivascular treatment was used for these studies because RWJ-58259 is not orally active. Required intravenous infusion rates were not practical via minipump. This particular polymer has been successfully used for slow release of compounds. Since the material is absorbed slowly, we anticipated that RWJ-58259 would be released slowly over a period of time. Release kinetics were not performed for these studies. However, material tends to stay where placed and compound concentrations are expected to be high locally, likely resulting in significant levels reaching the luminal edge of the vessel. Fourteen days after injury, rats were anesthetized and perfusion-fixed with buffered formalin. Eight left carotid tissue sections (5 μm, 100 μm apart) were stained for elastin and used for morphometric analysis (Cheung et al., 1999). Medial and intimal area and thickness were measured using image analysis software. Percent stenosis was computed as intimal area as a percentage of the total area within the internal elastic lamina.

Data Analysis. All results are presented as mean ± S.E. Statistical analysis was performed either by the Student's t test or one-way analysis of variance where indicated. Mean values were considered statistically significant when $P < 0.05$.

Results

RWJ-58259 Is a Potent PAR-1 Antagonist. We recently described a series of indole-based peptide mimetics represented by RWJ-56110, which inhibits thrombin-induced PAR-1 activation in human platelets and vascular cells (Andrade-Gordon et al., 1999). Replacement of the indole template with an indazole template afforded an improved chemical series, represented by RWJ-58259 (Fig. 1). We selected this PAR-1 antagonist for animal studies because of its good potency, PAR-1 selectivity, and particularly, in vivo safety profile.

RWJ-58259 inhibited 0.15 nM α-thrombin and 2 μM SFLLRN-induced aggregation of human gel-filtered platelets with IC_{50} values of 0.37 ± 0.07 μM (n = 12) and 0.11 ± 0.01 μM (n = 9), respectively. The PAR-1 action of RWJ-58259 was verified by its failure to inhibit human gel-filtered platelet aggregation stimulated by either collagen or the thromboxane mimetic U46619. In addition, RWJ-58259 effectively inhibited human platelet-rich plasma aggregation induced by 7.5 nM α-thrombin (IC_{50}, 8.0 ± 2.0 μM, n = 3). The higher IC_{50} observed for RWJ-58259 in platelet-rich plasma studies most likely reflects both the elevated thrombin concentration required to activate platelets in plasma due to endogenous thrombin inhibitors as well as increased binding of RWJ-58259 to plasma proteins. At elevated concentrations of

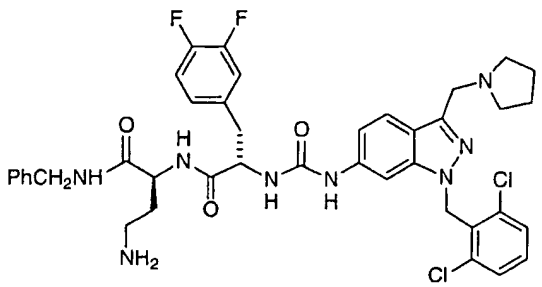

Fig. 1. Chemical structure of indazole-based peptide-mimetic RWJ-58259.

thrombin (e.g., 10–30 nM) with either human gel-filtered platelets or platelet-rich plasma, as observed previously for RWJ-56110 (Andrade-Gordon et al., 1999), RWJ-58259 became refractory in a thrombin dose-dependent manner, reflecting the dual PAR system on human platelets. The PAR-1 selectivity of RWJ-58259 was confirmed in the same, detailed fashion as described for RWJ-56110 (results not shown) (Andrade-Gordon et al., 1999).

In rat aortic smooth muscle cells, RWJ-58259 was found to inhibit α-thrombin-induced calcium mobilization (IC_{50} = 0.07 ± 0.01 μM, n = 4) and proliferation (IC_{50} = 2.3 ± 0.0 μM, n = 2). RWJ-58259 also blocked α-thrombin-induced interleukin-6 release from human aortic smooth muscle cells (IC_{50} = 3.6 ± 2.3 μM, n = 2). By contrast to human platelets, full antagonism of thrombin's action was observed in these vascular cells at high thrombin concentrations (e.g., 200 nM; results not shown). The ability of RWJ-58259 to inhibit signaling and function in smooth muscle cells, independent of thrombin concentration, is reflective of PAR-1 being the only thrombin-sensitive receptor on these cells (Andrade-Gordon et al., 1999).

Effects of RWJ-58259 on Guinea Pig Platelets. Guinea pig platelets have been widely used to test for PAR-1 action in platelet aggregation because they are responsive to the PAR-1-activating peptide SFLLRN-NH_2 (Connolly et al., 1994; Derian et al., 1995), which indicates the presence of functional PAR-1 on the cell surface. Since guinea pig platelets have a lot in common functionally with human platelets, we chose this small animal to explore PAR-1 antagonism in vivo. Our previous findings with the PAR-1 antagonist RWJ-56110 (Andrade-Gordon et al., 1999) confirmed the dual PAR activation system on human platelets. Given this background, we evaluated RWJ-58259 with guinea pig platelets for a similar mode of action. RWJ-58259 inhibited 7.5 nM α-thrombin-induced platelet-rich plasma aggregation with an IC_{50} of 7.4 ± 1.4 μM (n = 5), consistent with results from human platelet-rich plasma studies. Moreover, at a 10-fold higher concentration of α-thrombin, no inhibition was observed up to 100 μM RWJ-58259. In contrast, RWJ-58259 fully inhibited supramaximal concentrations of SFLLRN-NH_2 (100 μM)-mediated aggregation at a concentration of 10 μM. These results are indicative of another thrombin-sensitive receptor on guinea pig platelets in addition to PAR-1, as noted previously for human platelets. Because of the similarities between the in vitro behavior of RWJ-58259 in both human and guinea pig platelets, we considered this to be a suitable animal model for the investigation of PAR-1 physiology.

RWJ-58259 Inhibits ex Vivo Guinea Pig Platelet Aggregation. RWJ-58259 was first evaluated in a model of ex vivo platelet-rich plasma aggregation to determine the appropriate in vivo concentration ranges for further studies. RWJ-58259, administered to guinea pigs (0.3–3 mg/kg), inhibited α-thrombin-induced platelet-rich plasma aggregation in a concentration-dependent manner (Fig. 2). However, as α-thrombin concentrations were raised, RWJ-58259 became less effective, indicating that its ability to inhibit thrombin-mediated responses in vivo is dependent on the thrombin concentration. RWJ-58259-inhibited SFLLRN-induced aggregation under all conditions (results not shown). A dose of 10 mg/kg was chosen for further evaluations based on these results as well as pilot studies with earlier analogs including

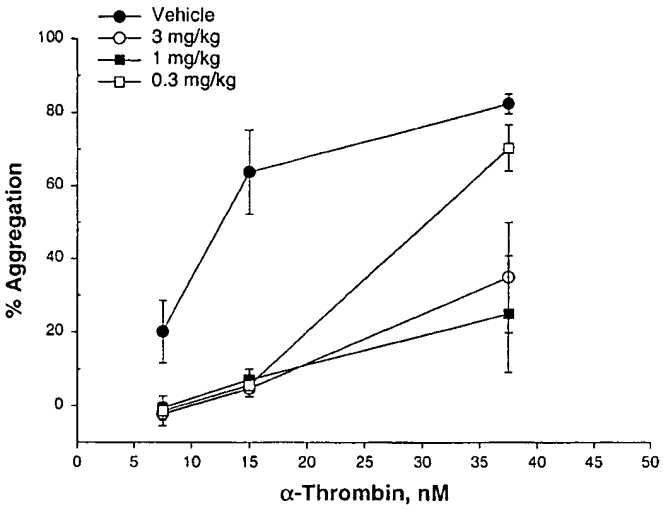

Fig. 2. Effects on ex vivo platelet-rich plasma aggregation after i.v. administration of RWJ-58259. Platelet-rich plasma aggregation in response to increasing concentrations of thrombin was inhibited after 0.3, 1, and 3 mg/kg of RWJ-58259.

RWJ-56110, which was ineffective at 6 mg/kg. This dose was the maximally tolerated intravenous dose for RWJ-58259.

Guinea Pig Arteriovenous Shunt Thrombosis Model. In this thrombosis model, a thrombus comprised of platelets, fibrin, and red blood cells forms on a section of cotton thread placed in an extracorporeal shunt between the carotid artery and jugular vein. Antithrombotic efficacy is indicated by decreases in the weight of thrombus accumulated during 15 min of exposure to flowing blood. Intravenous administration of RWJ-58259 (10 mg/kg) did not reduce thrombus weight (42 ± 4 mg, n = 2) when compared with a control group (43 ± 2 mg, n = 15) even though α-thrombin and SFLLRN-induced platelet-rich plasma aggregation were completely inhibited (Fig. 3A). The direct thrombin inhibitor inogatran (1 mg/kg, i.v.) or aspirin (100 mg/kg, i.v.) significantly decreased thrombus weight to 18 ± 3 mg (n = 6) and 16 ± 1 mg (n = 4), respectively. In a separate group of guinea pigs, RWJ-58259 was administered directly into the shunt just proximal to the thread in a protocol to maximize potential antithrombotic efficacy. An infusion rate of 0.1 mg/kg/min (2.0 mg/kg total dose) decreased thrombus weight slightly from a control of 35 ± 2 mg (n = 5) to 28 ± 4 mg (n = 4) (Fig. 3B). Increasing the infusion rate to 0.3 mg/kg/min (6.0 mg/kg total dose) further decreased thrombus weight to 24 ± 4 mg (n = 3). In these studies, the drug concentration (22 μM and 66 μM, respectively) was high enough to effectively inhibit α-thrombin and SFLLRN-induced platelet-rich plasma aggregation. Higher doses of RWJ-58259 could not be evaluated due to a combination of drug solubility and infusion volume. Bleeding times and activated clotting times were not changed. By comparison, administration of inogatran directly into the shunt at a rate of 0.01 mg/kg/min (0.2 mg/kg total dose) significantly decreased thrombus weight to 14 ± 2 mg (n = 3).

Guinea Pig Photoactivation Thrombosis Model. Intravascular photoactivation of the dye Rose Bengal with a green, heat-filtered xenon light results in endothelial damage that stimulates platelet adhesion to the vessel wall and generalized initiation of a platelet-rich thrombo-occlusive event. Antiplatelet agents and to a lesser extent, anticoagulants are

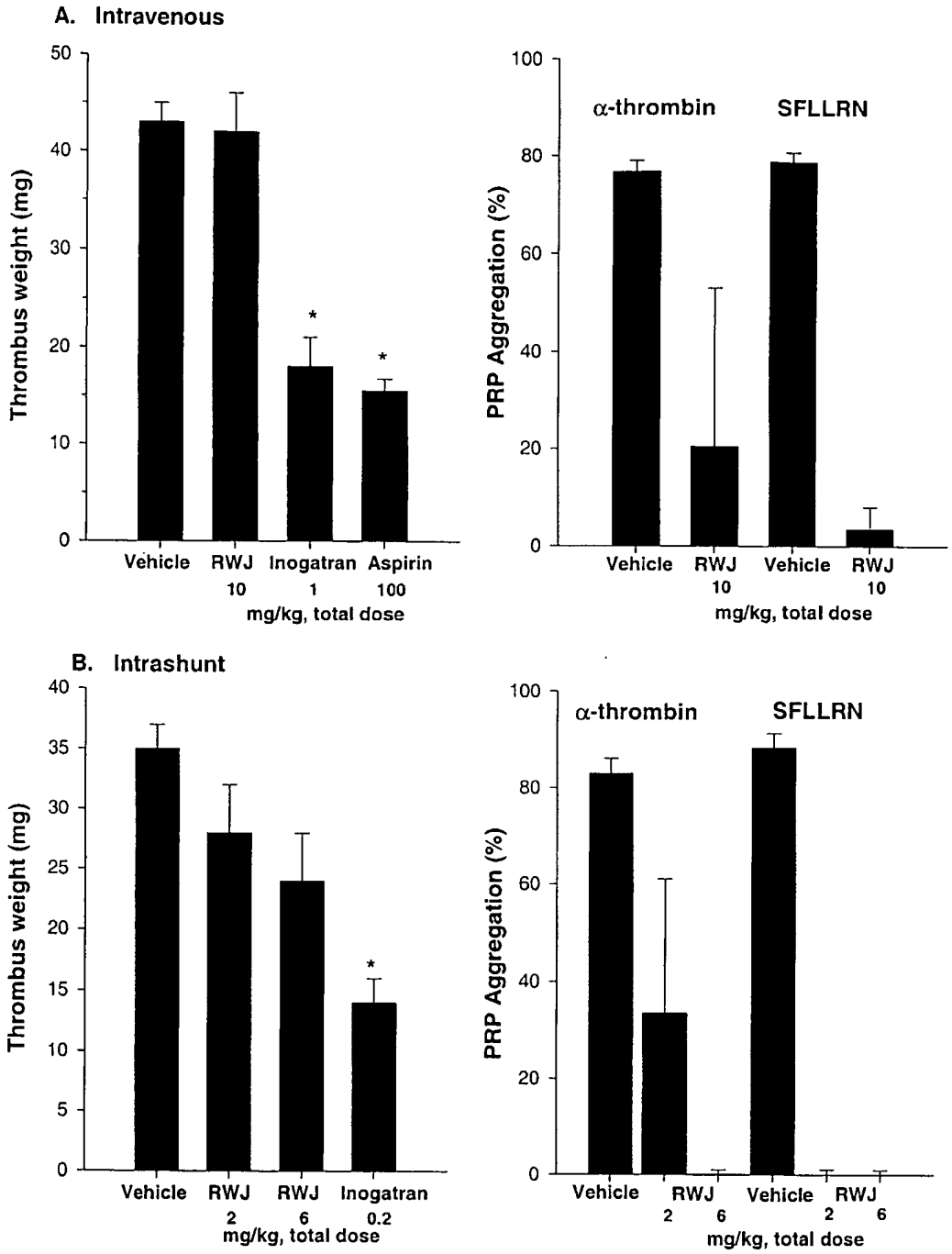

Fig. 3. Effect of RWJ-58259 (RWJ) in the guinea pig A-V shunt antithrombotic model. A, intravenous administration of RWJ-58259; B, intrashunt administration of RWJ-58259. Left panels, antithrombotic efficacy measured as reduction of thrombus weight compared with vehicle control; right panels, ex vivo platelet-rich plasma aggregation induced by 35 nM α-thrombin or 50 μM SFLLRN-NH_2. *$P < 0.05$ compared with vehicle treated by Student's paired t test.

effective in this model. Figure 4 (top panel) depicts carotid arterial perfusion and occlusion, as measured by Doppler flow, with each bar representing an individual animal. Initial occlusion times for the saline- and dextrose-treated animals averaged approximately 15 min. In all vehicle-treated animals except one, the arterial occlusion remained stable, whereas in the drug-treated groups the occlusion was unstable with intermittent flow observed over 30 min. At 1 mg/kg of the thrombin inhibitor r-hirudin, three of six treated animals were flowing at 30 min, and two of six did not experience occlusion. Two of eight RWJ-58259 treated animals were flowing at 30 min and one of eight did not experience occlusion. Total cumulative perfusion times (Fig. 4, middle panel) were significantly extended by r-hirudin at 1 and 3 mg/kg. RWJ-58259 at 10 mg/kg tended to increase perfusion times but this effect was not significant. RWJ-58259 significantly inhibited thrombin and SFLLRN-NH_2-induced platelet aggregation ex vivo (Fig. 4, bottom panel). Aggregation to low concentrations of α-thrombin (7–25 nM) was significantly inhibited, whereas aggregation at higher concentrations was much more variable and was determined not to be significantly different from that in the untreated animals. Aggregation to SFLLRN-NH_2 was completely inhibited by RWJ-58259 at all concentrations evaluated.

Guinea Pig Platelet PAR Profile. In vitro studies with our selective PAR-1 antagonist RWJ-58259 indicated the

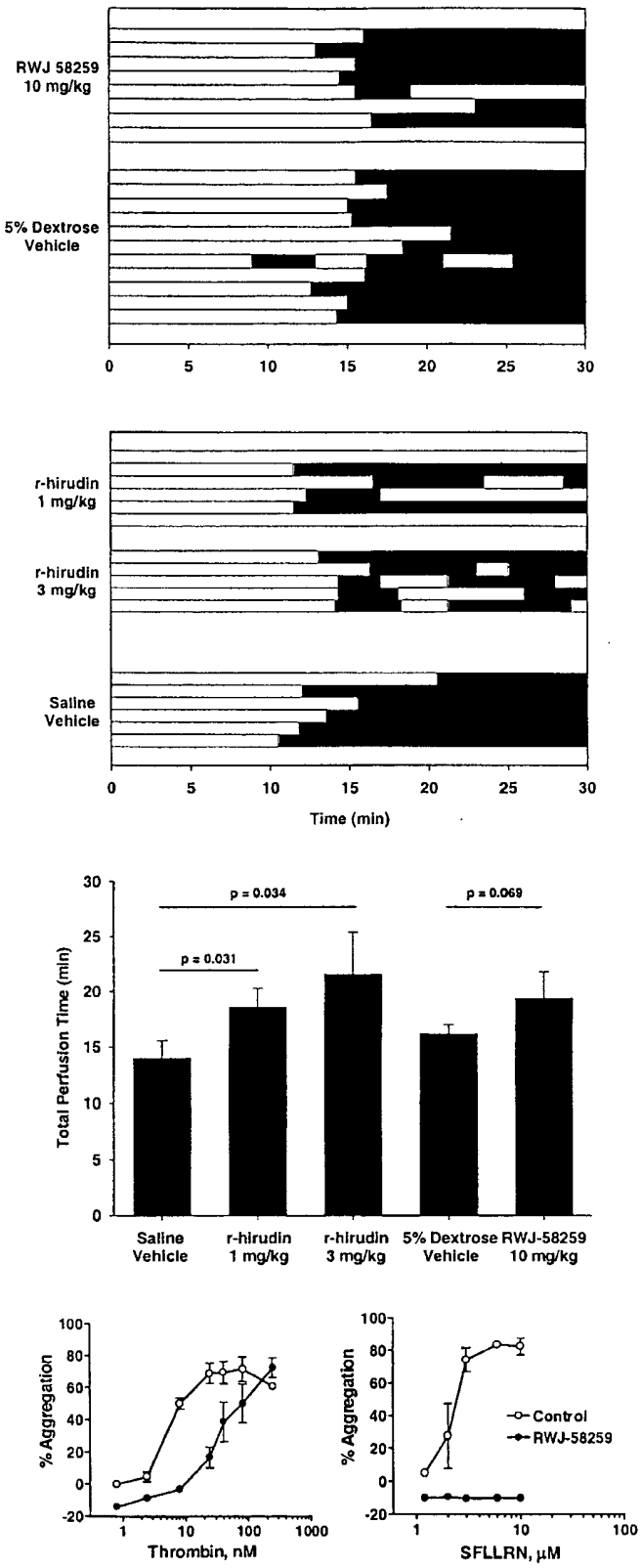

Fig. 4. Effect of RWJ-58259 in the guinea pig photoactivation model. Top panel, carotid arterial perfusion with each bar representing individual animals treated with vehicle, r-hirudin, or RWJ-58259; light shaded areas, flow; dark areas, no flow. Middle panel, average total, cumulative, perfusion time for each group. $^*P < 0.05$ compared with vehicle control by Student's paired t test. Bottom panel, ex vivo aggregation of platelets prepared from control (○, $n = 3$) and RWJ-58259-treated (●, $n = 6$) guinea pigs.

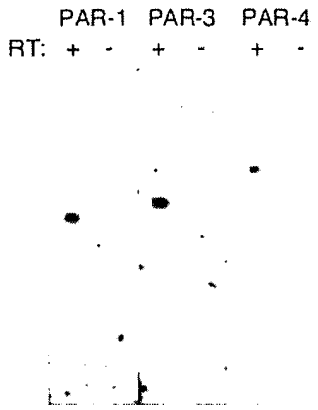

Fig. 5. PAR profile in guinea pig platelets. RT-PCR was used to examine the presence of PAR-1, PAR-3, and PAR-4 mRNA in guinea pig platelets. The predicted sizes of the amplicons, detected following hybridization with the appropriate nested primer probe, are as follows: PAR-1, 395 bp; PAR-3, 468 bp; and PAR-4, 746 bp.

presence of more than one thrombin receptor on both human and guinea pig platelets. Furthermore, results from the guinea pig in vivo thrombosis models suggested that another thrombin receptor, possibly PAR-4, plays a role in platelet-dependent thrombosis. Therefore, it was necessary to characterize the thrombin-receptor profile of guinea pig platelets. Although PAR-1 has been cloned from several species, only human and murine PAR-4 have been cloned and characterized (Kahn et al., 1998; Xu et al., 1998). Thus, we tested human PAR-1 (SFLLRN-NH_2)-, human PAR-4 (GYPGQV-NH_2)-, and murine PAR-4 (GYPGKF-NH_2)-activating peptides on human, rat, and guinea pig platelets. Whereas the human PAR-1 and PAR-4 peptides induced human platelet aggregation and the human and murine PAR-4 peptides induced rat platelet aggregation (no PAR-1 in rat platelets), only the PAR-1 peptide induced guinea pig platelet aggregation (not the PAR-4 peptides; results not shown). This outcome agrees with a recent communication by Nishikawa et al. (2000), in which washed guinea pig platelets do not respond to the murine PAR-4 peptide up to a concentration of 1 mM. To follow up on this observation, we examined the constitution of PARs in isolated guinea pig platelets by RT-PCR and were able to detect the mRNAs corresponding to PAR-1, PAR-3, and PAR-4 (Fig. 5). The apparent paradox of guinea pig platelets containing the message for PAR-4, but failing to respond to the human or murine PAR-4 peptides, was probed by isolating the guinea pig PAR-4 gene and characterizing the second exon.[1] Like the genomic organization of other so-characterized PARs, exon 2 of the guinea pig PAR-4 gene contains the coding sequences of the entire receptor, without the initiation codon and signal sequence. Interestingly, sequence analysis revealed that guinea pig PAR-4 contains the activation motif SFPGQA, which diverges from the motifs in human (GYPGQV) or murine (GYPGKF) PAR-4. We synthesized SFPGQA-NH_2 and found that it does induce the aggregation of guinea pig platelets with an EC_{50} of 131 μM. This result illustrates a notable flexibility in the evolution of the PAR-4 gene. In the final

[1] A. Darrow, C. Derian, M. Addo, and P. Andrade-Gordon, manuscript in preparation.

analysis, guinea pig platelets possess two functional thrombin-responsive systems, PAR-1 and PAR-3/PAR-4.

Effects of RWJ-58259 in a Rat Restenosis Model. Since α-thrombin-mediated vascular smooth muscle cell responses associated with vascular injury (inflammatory cytokine release and cell proliferation) were inhibited by RWJ-58259, this agent would be a good candidate to assess the role of PAR-1 in a rat balloon angioplasty model of vascular injury. Furthermore, since rat platelet aggregation stimulated by α-thrombin was not inhibited by RWJ-58259, confirming the lack of PAR-1 on these cells as well as the PAR-1 selectivity of RWJ-58259, this in vivo model would reflect effects directly on the vasculature. Perivascular treatment (1, 5, 10 mg) with RWJ-58259 produced dose-related reductions in intimal area and thickness, and a decrease in percent stenosis (Table 1), which became statistically significant at the 10 mg dose. Medial area and thickness were not changed, resulting in a significant reduction in the intimal to medial ratio. There was no evidence of an effect on remodeling. There was a trend toward increased lumen area at the 10 mg dose, but this was not significant. There was no significant difference in the vessel size among the treatment groups. An example of the effect of RWJ-58259 on vascular injury is shown in Fig. 6. Thus, there is a clear reduction in neointimal thickness in the section from a rat treated with RWJ-58259 compared with a section from a rat treated with vehicle. These results indicate that inhibition of thrombin-induced activation of PAR-1 in vivo can reduce the vascular injury response.

Discussion

The thrombin receptor PAR-1 has been implicated in a variety of cellular events mediated by thrombin, including those associated with thrombosis and vascular injury. In this report, we have demonstrated that PAR-1 is involved in the restenotic events associated with balloon angioplasty in rats by using a potent, selective PAR-1 antagonist, RWJ-58259. Furthermore, results with RWJ-58259 in two different guinea pig thrombosis models reveal that PAR-1 may partially mediate platelet-dependent thrombus generation; however, there are serious concerns about the suitability of this, and other, species for such antithrombotic studies.

Antithrombotic Effect of RWJ-58259. The presence of divergent thrombin-receptor profiles for platelets of different species was first recognized in studies employing the PAR-1 agonist peptide SFLLRN (Connolly et al., 1994; Derian et al., 1995). Platelets isolated from the blood of humans, primates, and guinea pigs, but not rabbits, rodents, and dogs, were responsive to SFLLRN, although all of the species responded to thrombin. Based on the species studies, we reasoned that the guinea pig would provide an appropriate small-animal model to assess platelet PAR-1-dependent responses associated with thrombosis. In both models evaluated, inhibition of thrombin's proteolytic activity resulted in significant antithrombotic effects, confirming a significant role for thrombin-mediated thrombus formation. Our results with RWJ-58259 revealed just a modest effect on thrombus formation in the two guinea pig models, raising the distinct possibility that PAR-1 is not a significant contributor to platelet thrombus formation. Our in vitro and in vivo platelet aggregation results with RWJ-58259 indicated that it is an effective antagonist of guinea pig PAR-1; however, its effectiveness was dependent on thrombin concentration. Complete antagonism of thrombin in vitro was achieved at low thrombin concentrations, but the effect diminished as the thrombin levels rose above 10 nM. Thus, we hypothesized that another thrombin-responsive receptor existed on guinea pig platelets.

Three thrombin receptors, PAR-1, PAR-3, and PAR-4, have been described and the PAR profiles of human and murine platelets have been reasonably well defined (Vu et al., 1991; Ishihara et al., 1997; Xu et al., 1998). On the basis of studies with PAR-3-deficient mice, it appears that a dual thrombin receptor system (PAR-3/PAR-4) exists on the platelets of wild-type mice (Kahn et al., 1998). However, human platelets do not express PAR-3 and thus PAR-1 was considered to be the only thrombin receptor on these cells. The discovery of human PAR-4 then suggested that human platelets do have a dual thrombin receptor system (PAR-1/PAR-4) (Xu et al., 1998). The presence of PAR-4 on human platelets is consistent with the loss of thrombin antagonist activity with our PAR-1 antagonists, RWJ-56110 and RWJ-58259 at elevated thrombin concentrations (Andrade-Gordon et al., 1999). Since the activity of RWJ-58259 was similar in isolated human and guinea pig platelets, we hypothesized that the results of our in vivo thrombosis models reflected a dual thrombin receptor system, PAR-1 and PAR-4, on guinea pig platelets.

Therefore, we sought to determine the PAR profile of guinea pig platelets, first by agonist peptide studies, then by RT-PCR. Surprisingly, our results indicate a triple PAR expression pattern with the presence of PAR-1, PAR-3, and PAR-4. This result raises important questions about the complex interactions of the different PARs during thrombus formation in different species and ultimately, the significance of PAR-4 activation in human thrombotic disease. The interaction of PAR-3 and PAR-4 was elegantly described by Nakanishi-Matsui et al. (2000), who demonstrated that PAR-3 serves as a cofactor for PAR-4, thereby increasing the thrombin sensitivity of PAR-4 by as much as 10-fold. The coordinated action of PAR-3/PAR-4 appears to mirror the action of

TABLE 1
Effects of adventitially administered RWJ-58259 on vascular injury in response to balloon angioplasty in the carotid artery of rats

Group	No.	Area, mm^2			Thickness, μM			Vessel Dimensions, mm^2	
		Intima	Media	% Stenosis	Intima	Media	I/M	Lumen	Vessel
Vehicle	10	0.129 ± 0.005	0.123 ± 0.006	44 ± 3	77 ± 5	58 ± 2	1.35 ± 0.09	0.231 ± 0.026	0.480 ± 0.028
1 mg	9	0.118 ± 0.010	0.118 ± 0.007	38 ± 2	68 ± 5	55 ± 2	1.24 ± 0.09	0.258 ± 0.019	0.482 ± 0.026
5 mg	9	0.110 ± 0.008	0.113 ± 0.007	36 ± 3	63 ± 4	53 ± 2	1.19 ± 0.09	0.239 ± 0.025	0.447 ± 0.035
10 mg	8	0.084 ± 0.007*	0.121 ± 0.006	26 ± 3*	45 ± 5*	54 ± 2	0.83 ± 0.10*	0.320 ± 0.031	0.507 ± 0.034

I/M, intima/media.
*$P < 0.05$, significantly different from vehicle by one-way analysis of variance.

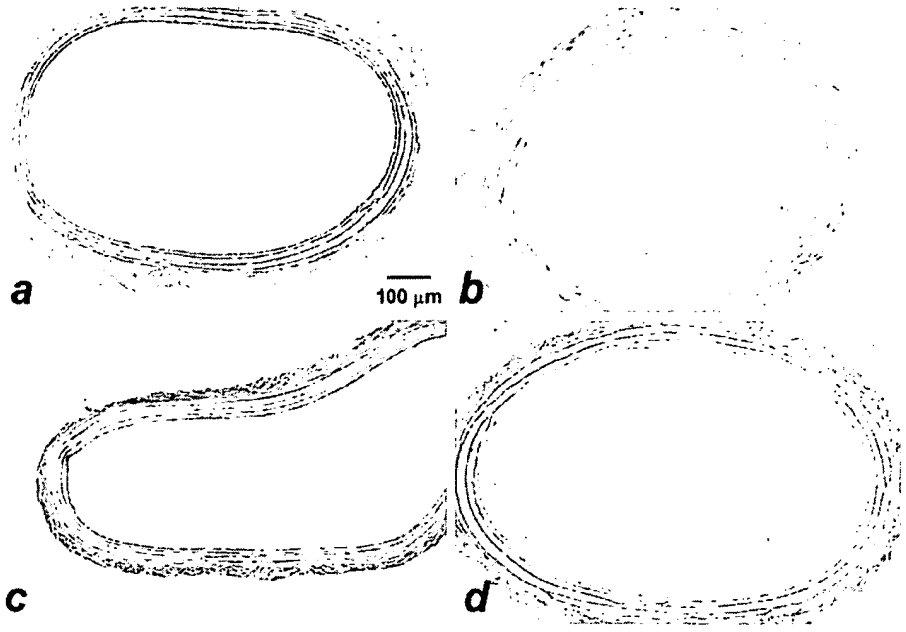

Fig. 6. Examples of rat carotid artery changes 14 days after vascular injury. A, uninjured, vehicle-treated; B, injured, vehicle-treated; C, uninjured, RWJ-58259-treated; and D, injured, RWJ-58259-treated. Scale bar, 100 μm.

PAR-1 with respect to thrombin sensitivity. The expression of all three PARs in guinea pig platelets suggests that two equally responsive thrombin systems, PAR-1 and PAR-3/PAR-4, exist there. The lack of significant antithrombotic activity for RWJ-58259 in the two guinea pig thrombosis models can be explained by the occurrence of thrombin-dependent platelet activation via PAR-3/PAR-4 during complete PAR-1 blockade. Our results indicate that guinea pigs may not be a suitable animal model for evaluating PAR-1 antagonists as potential antithrombotic drugs for humans.

The physiological role of this PAR redundancy may be a protective system to assure effective, rapid platelet aggregation during severe vascular injury, when concentrations of thrombin would be explosively elevated. Because of the complexity of multiple PARs, it has been difficult to dissect the contributions of individual PARs to the process of thrombosis, and this may have prevented the development of a PAR-1 antagonist as a potential therapeutic agent. The potential significance of PAR-4 activation in human clinical disease remains to be determined. Future studies in nonhuman primates, whose platelet PAR profile is similar to that of humans (unpublished observation), should provide a better means to evaluate the antithrombotic efficacy of selective PAR-1 antagonists.

Antirestenotic Action of RWJ-58259. Vascular injury associated with angioplasty procedures results from both thrombotic and restenotic components. While our results with RWJ-58259 in the thrombosis models did not conclusively determine the impact of PAR-1 antagonism on thrombotic processes, RWJ-58259 showed significant inhibition of neointimal thickening in the rat model of vascular injury, consistent with a direct effect on PAR-1-mediated vascular smooth muscle function. These results are highly significant, since rat platelets are fully responsive to thrombin through PAR-3/PAR-4 activation. Our results are consistent with a recent study that showed a reduced vascular injury response in rats treated with an antibody to PAR-1 (Takada et al., 1998). We have also found that the vascular injury response is reduced in mice deficient in PAR-1 compared with wild-type mice (Cheung et al., 1999).

PAR-1 is up-regulated in vascular smooth muscle cells in response to vascular injury in animal models (Wilcox et al., 1994; Cheung et al., 1999) and in human atherosclerotic coronary arteries (Nelken et al., 1992). This up-regulation is associated with proliferating cells. Thus, the effectiveness of PAR-1 antagonism in reducing vascular injury may be the result of inhibition of PAR-1-mediated vascular smooth muscle proliferation (McNamara et al., 1993). Consistent with this view, RWJ-58259 effectively inhibited thrombin-induced calcium mobilization and proliferation in rat aortic smooth muscle cells. Thrombin levels are also greatly increased at sites of vascular injury (Hatton et al., 1989; Harker et al., 1995). Although thrombin inhibitors have reduced vascular injury responses in several animal models (Heras et al., 1990; Barry et al., 1996; Gerdes et al., 1996), initial clinical trials have been unable to show the effectiveness of thrombin inhibition in vascular injury (Serruys et al., 1995; Burchenal et al., 1998). This observation may derive from inadequate treatment regimens. Alternatively, there may be some advantage to the specific blockade of PAR-1 as opposed to the inhibition of all of thrombin's many actions with a direct enzyme inhibitor.

In summary, we were unable to ascertain the antithrombotic potential of a PAR-1 antagonist in guinea pig models of thrombosis because of interference from the PAR-3/PAR-4 system present on guinea pig platelets. Thus, a determination of possible antithrombotic utility preclinically would require studies that surmount the species issue, such as through the use of primate models. However, our results with RWJ-58259 in rats indicate that selective antagonism of PAR-1 can significantly attenuate restenosis following balloon angioplasty. Accordingly, inhibition of PAR-1 may have therapeutic potential in human vascular injury.

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